STRUCTURE-FUNCTION RELATIONSHIP AND REGULATION OF ORGANIC ANION TRANSPORTERS (OATS)

by

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ABSTRACT OF THE DISSERTATION

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The organic anion transporter (OAT) family has been identified to express in multiple human organs, such as kidney, liver, brain and placenta. They mediate the absorption, distribution, and excretion of a diverse array of environmental toxins, and clinically important drugs and therefore are critical for the survival of mammalian species. My research work has focused on the elucidation of the structure-function relationship and regulation of OATs.

It was known that glycosylation of proteins play critical roles in many cell functions. My studies have identified that the addition/acquisition of oligosaccharides but not the processing of the added oligosaccharides plays a critical role in the targeting of hOAT4 to the plasma membrane. Processing of added oligosaccharides from mannose-rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates.

According to the alignment of several OATs isolated from different species, my studies have explored several conserved important residues on human organic

anion transporter 4 (hOAT4), which affect transporter function by impairing the trafficking of the transporter to the cell surface or its binding ability for the substrates.

I have also characterized the organic anion transport system in rat and human placental cell lines, Hrp1 and Bewo, which has great significance in establishing the useful *in vitro* models for the studies of the transfer of nutrients and drugs between mother and the fetus and providing important information for the understanding of placental physiology and diseases.

Furthermore, my studies have explored that organic anion transporter function can be regulated by protein kinase C and placental hormones. I have investigated the differences between the interaction of PDZ proteins with hOAT4 in kidney cells and that in placental cells as well. My data indicated that the interacting partners of hOAT4 in placenta may be different from that in kidney.

The information obtained from my studies will be great significance to the development of clinically useful drugs and to the advancement of our understanding of the molecular, cellular, and clinical bases of renal, hepatic, neurological and fetal toxicity and diseases.

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I also want to thank my committee members, Dr. Tamara Minko, Dr. Bo Michiniak and Dr. Zui Pan, who have given me insightful suggestions and great helps in my research work.

Finally I want to dedicate my work to my parents, my husband and my daughter, who are the people standing behind me and supporting me for all these years.

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Introduction

Kidney is a key organ of human body. They are two small organs located near the vertebral column at the small of the back. The left kidney lies a little higher than the right kidney. They are bean-shaped, about 4 in. (10 cm) long and about 21/2 in. (6.4 cm) wide. The basic functional unit of the kidney is the nephron (Fig. 1). There are more than a million nephrons within the cortex and medulla of each normal adult human kidney. Nephrons can filter the blood under pressure, secrete some unneeded and then reabsorb some necessary fluid and molecules back into the blood. The proximal tubule is the major site for toxin elimination, which expresses an epithelial transport system. Then the fluid flows from the nephron into the collecting ducts, which is responsible for water conservation.

The main functions of human kidney is to maintain total body homeostasis, such as fluid volume, acid-base balance, electrolyte concentration and hormone levels, through in charge of the elimination of the toxic substances in the human body. These toxic substances can be either endogenous or exogenous, such as from environment, drugs or human disease state. Organic anion (OA) is an important category of the toxic substances.

Organic anion (OA) is the chemically heterogeneous substance, which contains the carbon backbone and a negative charge at physiological pH. A wide range of drugs, environmental or endogenous toxins and their metabolites are OAs, such as penicillin G, glutarate. It turned out to be that these OAs are unable to diffuse through the lipid bilayer of renal proximal tubular cells freely, so there must be some specific membrane transport proteins involved in this process.

Up to date, there are thousands of transporter proteins have been identified and cloned. Among them, organic anion transporter (OAT) is an essential family of transport proteins, which plays a critical role in renal elimination and detoxification. OAT belongs to the amphiphilic solute transporter superfamily *Slc22a* of the Major Facilitator Superfamily (1-3). It has been identified that many OAs can be interacted with or transported by OATs in human body. The substrates of OATs include many widely used clinical drugs, such as β-lactam and sulfonamide antibiotics, nonsteroidal anti-inflammatory drugs (NSAID), diuretics, antitumor drugs, angioensin converting enzyme inhibitors and antiviral agents (Table 1) (4-6).

There are several members of OATs have been cloned: OAT1, OAT2, OAT3, OAT4, OAT5, OAT6, OAT7 and urate transporter 1 (URAT1). They express in various tissues, except for OAT6 and OAT7, OAT1-5 and URAT1 all have been identified expressing in kidney (7-15).

Selected species, sex, and tissue distribution of OATs

OAT1 (Slc22a6) was originally cloned as NKT (10, 16-18). It has been identified expressing in various species, such as human, mouse, rat and C. *elegans* (16-24). Human, rat and mouse OAT1 mainly expressed at the basolateral membrane of kidney proximal tubular cells and at less extent in brain (10, 16). Recently mOAT1 has also been detected also expressing in olfactory mucosa (11). It was found out that mouse OAT1 expressed more in male mouse kidney than in female mouse kidney (10).

OAT2 (Slc22a7), alternatively named as novel liver-specific transporter (NLT), was originally isolated from rat liver (12). Human OAT2 was identified highly expressed in liver and weakly in kidney (25). Interestingly, mouse and rat OAT2 has a sex- and species-related expression (26-28). mOAT2 is abundantly expressed in the male mouse kidney, while it was predominantly expressed in both the liver and the kidney of female mouse (kidney > liver) (27). In male rat, rOAT2 is higherly expressed in the liver than in the kidney, while in female rat, it is largerly expressed in the kidney than in the liver (26).

OAT3 (Slc22a8) was first cloned from rat brain (9), later found to be higherly expressed in the kidney than in the liver and eye (9, 26). However, human OAT3 (hOAT3) was largely expressed at the basolateral membrane of proximal tubules of kidney and also in brain and skeletal muscles, but not in liver (29, 30). mOAT3 was reported to be abundant in kidney, weakly detected in choroid plexus (31) and also in developing bone by *in situ* hybridization analysis (7). In male rat, OAT3 is found to be more largely expressed in kidney than in liver, while in female rat, OAT3 is majorly expressed in kidney (26, 32, 33).

OAT4 (Slc22a11), different from other OATs, has only been found in human and is largely localized at the basal surface of syncytiotrophoblast cells of placenta and little at the apical membrane of the renal proximal tubular cells (34, 35). There was a recent study showed that hOAT3 and hOAT4 also expressed in human adrenocortical cells, which indicated that OATs may have more presences in other tissues that people have not detected (36).

The newly isolated mouse and rat OAT5 (Slc22a10), which are not homologs to hOAT5 (25), have been identified to express mainly at the apical membrane of the kidney proximal tubular cells (13, 37, 38).

Recently mouse OAT6 has been cloned in olfactory mucosa and also found to be weakly expressed in testis (11).

Most recently there was a paper published on Hepatology (14), which has characterized the transport function of a Novel liver-specific organic anion transporter OAT7, which can operate the exchange of sulfate conjugates for short chain fatty acid butyrate.

Another important member of OAT is the urate transporter 1 (URAT1) (Slc22a12), which was identified on the apical membrane of human, rat and mouse proximal tubular cells in the kidney (39, 40). For mouse URAT1, male mouse has higher expression than female ones (39, 40).

Table 2 summarizes the tissue distribution of each OATs.

Substrate specificity of OATs

Substrate specificity is a critical character of transporters. OATs can interact with thousands of organic anions; many of them are clinically important drugs, endogenous or environmental toxins and their metabolites. Although the OATs have wide range substrate spectra and share a lot of common substrates, they have their own unique selectivity for certain substances (41).

A common substrate for OAT1, OAT2, OAT3, OAT4, OAT5, ochratoxin A (OA), is a mycotoxin produced by several species of the mould genera aspergillums and

penicillium, and has been proven to be a teratogen, carcinogen and nephrotoxin implicated in animals and possibly human nephropathies (42).

Para-aminohippuric acid (PAH), an end product of the metabolism of aromatic amino acids produced in the body in small amounts, is a prototypical substrate for hOAT1, hOAT2 and hOAT3, but has low affinity to hOAT4 and mOAT5 (42).

Dicarboxylates, such as α-ketoglutarate and glutarate, can be transported by hOAT1, hOAT2, hOAT3, hOAT4 (42).

It was reported that hOAT1 and rOAT2 can both interact with methotrexate (a popular anti-tumor drug) and salicylate (one non-steroidal anti-inflammatory drug) (41).

hOAT1, hOAT2 and hOAT4 can mediate high-affinity transport of prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), while hOAT2, hOAT3, hOAT4 and hOAT7 have high substrate selectivity for estrone-3-sulfate (ES), estrone sulphate and dehydroepiandrosterone sulfate (DHEAS) (14, 42).

Furthermore, individually speaking, recent study showed that hOAT1 can interact with cysteine (Cys) S-conjugate of methylmercury (CH(3)Hg-Cys) (43); hOAT2 has been identified to transport bumetanide, allopurinol, 5-fluorouracil, paclitaxel and L-ascorbic acid (44); hOAT3 is reported to be a potent transporter for cephalosporin antibiotics, such as cephaloridine, cefdinir, cefotiam, ceftibuten, cefaclor, ceftizoxime, cefoselis and cefazolin (45). The newly study showed that OAT6 can transport ES, and the uptake of ES can be inhibited by probenecid, 2,4-dichlorophenoxyacetate ochratoxin A, salicylate, penicillin G,

p-aminohippurate and urate to various extent (46). And human OAT7 has been reported to transport short chain fatty acid butyrate (14).

URAT1 is unique among all the OATs, since it can reabsorb urate in kidney, but not uptake various typical substrates of OATs, such as PAH (39, 40). The weak acid, urate is an intermediate product of purine metabolism. In human or other higher primates, there is no uricase (urate oxidase) to oxidize purine into allanoin, so urate is the final product of purine metabolism. High concentration of urate in the blood will cause a lot of problem such as hyperuricemia, gout, hypertension and cardiovascular diseases. Besides urate, URAT1 can also transport a lot of monovalent anions, such as lactate, nicotinate (39, 40). A lot of URAT1 substrates have been used to reduce hyperuricemia, such as probenecid, NSAIDs and diuretic drugs, which can inhibit URAT1 in the body.

Table 3 gives out the substrate specificity of OATs.

Transport mode of OATs

It was reported that PAH uptake by OAT1 was sodium-independent and OAT1 plays a role as an organic anion/dicarboxylate exchanger (47). The inside > outside dicarboxylate (α-ketoglutarate) concentration gradient served as the driving force for PAH uptake by OAT1 (47). This kind of “tertiary” active transport mode is energetically uphill. Na^+-K^+-ATPase forms a blood-to-cell Na^+ gradient, which drives a cell-to-blood dicarboxylate gradient of the Na^+-dicarboxylate contransporter. Then this cell-to-blood Na^+ gradient can drive the OA substrate into the cells by the dicarboxylate/organic anion exchanger. The cascade of moving OA into cells against both its chemical concentration gradient and the

electrical potential requires consuming metabolic energy and existing of the Na+ gradient (Fig 2).

Although there was no clear conclusion about the transporter mode of OAT2, some data showed that OAT2 uptake was in a sodium-independent manner and could not be trans-stimulated by dicarboxylates (27, 28), which suggested that OAT2 transporter mode is probably not an organic anion/dicarboxylate exchanger.

Another important transporter expressed at the basolateral surface of proximal tubular cells, rOAT3, was identified to act as organic anion/dicarboxylate exchangers indirectly coupled with the sodium gradient, since the uptake of ES by rOAT3 in oocytes could be trans-stimulated by preloading with the dicarboxylate glutarate (GA) but not affected by disruption of Na^+ gradient (48).

Before it was thought hOAT4 might serve as a facilitated diffusion carrier, since the ES uptake of hOAT4 was sodium-independent and had no trans-stimulation (8). However, recently it was reported that the ES and glutarate uptake by hOAT4 can be trans-stimulated by each other, which indicated that hOAT4 is an organic anion/dicarboxylate exchanger (49).

Interestingly, mOAT5 mediated transport was neither cis-inhibited nor trans-stimulated by dicarboxylates, which suggested that mOAT5 is unlikely an organic anion/dicarboxylate exchanger (13); while another study showed that rat OAT5 was both cis-inhibited and trans-stimulated by dicarboxylates, which indicated that rOAT5 is an organic anion/dicarboxylates exchanger (37).

The recent studies showed that the uptake of ES by mouse OAT6 can be significantly trans-stimulated by glutarate, which showed that mOAT6 functions as an organic anion/dicarboxylate exchanger as well (46).

URAT1 was thought to work as an urate/organic anions exchanger, which means the organic anions accumulated in the cells binding to URAT1, could move down their electrochemical gradients into the tubular lumen and exchange urate into the cells (39).

Table 4 summarizes the transport mode of each OAT members.

Knockout mice models of OATs

Early up to 2002, Dr. Nigam's group first reported and characterized the knockout mice model of OAT3 (*Oat3*$^{-/-}$), in which there were no mRNA of OAT3 detected in kidney, liver or choroid plexus (CP) (33). These *Oat3*$^{-/-}$ mice showed to be healthy, fertile and had no morphological tissue abnormalities. But there was an impaired organic anion transport function detected in renal and choroid plexus epithelia, not in liver. The uptakes for taurocholate (TC), ES and *para*-aminohippurate (PAH) in renal slices of *Oat3*$^{-/-}$ mice were significantly reduced compared with that of the slices from the wild type littermates. Furthermore, there was no inhibitory sensitivity for bromosulfophthalein (BSP) and probenecid (PRO), which are two confirmed inhibitors of OAT3, in the uptake of TC on the renal slides of *Oat3*$^{-/-}$ mice. This significant impaired uptake and the lack of sensitivity to BSP and PRO in the uptake of TC indicated that the reduced uptake of TC in *Oat3*$^{-/-}$ mice compared with that of the wild type mice was contributed by OAT3 and OAT3 is largely responsible for the TC uptake in kidney. In choroid

plexus, the cellular accumulation of fluorescein (FL) was reduced in *Oat3*$^{-/-}$ mice, while the capillary accumulation of fluorescein-methotrexate was not changed compared with that of the wild type mice, this indicated that OAT3 played important roles in the disposition of FL in choroid plexus.

In another study, it showed that the uptake of PAH in the choroid plexus of *Oat3*$^{-/-}$ mice was significantly reduced compared with that of wild type littermates (31). But the facts that the uptake of ES was reduced only about one-third and there was no change with the uptake of TC in choroid plexus of *Oat3*$^{-/-}$ mice, suggested that there may be other organic anion transporters involved in the uptake of TC and ES, such as OATPs. These information proved us that OAT3 is responsible for the major disposition of PAH and partial uptake of ES in mice choroid plexus. All the data collected from *Oat3*$^{-/-}$ mice revealed us the essential role of OAT3 in body detoxification and also the disposition of organic anions in central nervous system (CNS).

Most recently OAT1 knockout mice (*Oat1*$^{-/-}$ mice) was generated (50). Similar to *Oat3*$^{-/-}$ mice, *Oat1*$^{-/-}$ mice has no morphological or physiological deficiency, but has the impairation of organic anion uptakes (50). It was identified that there were significant loss of the uptake of PAH, but no marked loss of the uptake of ES in the renal slices of *Oat1*$^{-/-}$ mice compared with those of the wild type littermates. And this observation was further confirmed by the predominant loss of PAH in the urinary excretion study *in vivo*, which indicated that OAT1 played the major role in the disposition of PAH *in vivo.* In addition, a lot of other substrates have been tested on the *Oat1*$^{-/-}$ mice model as well, including a lot of prescribed drugs,

xenobiotics and endogenous organic anions, such as 3-hydroxyisobutyrate, 3-hydroxybutyrate, 3-hydroxypropionate and benzoate. The renal excretion change of these substrates reflected the role of OAT1 played in the renal detoxification and elimination of these substances *in vivo*, which implied the possibility to use this mice model as one of the convenient clinical drug evaluation system in the future.

Until now there are no reports about the knockout mice models for other OATs, it will be really interesting to see what will happen to the knockout mice models of other OATs.

Developmental expression pattern of OATs in kidney

Over fifty years ago, people found that newborn infants excreted penicillin at a rate lower than that predicted by body weight alone (51). Later studies showed that this phenomenon is due to the reduced ability of the newborn kidney to eliminate drugs (52). This discovery caused the interests of scientists to study the ontogeny of renal OA transport system later on.

Studies of PAH secretion in human, dog, rat, rabbit and sheep showed the same pattern that PAH secretion is in newborns, increase over the first few weeks of neonatal life and then reduce to the adult level (53-59). This disproportion between OA secretion and renal mass increasing was involved with the maturation of OATs (52).

Northern blot and *in situ* hybridization analysis have been conducted to study the OATs maturation during murine development (10, 60, 61). Renal expression of mOAT1 can be detected at early embryonic day (Table 4), and continued to

increase through birth until reaching the highest level in 1 day postpartum, then reduced to adult level. Furthermore, this same pattern was also found in other OAT members, such as mOAT2, renal mOAT3 (12, 60). Since these OATs are almost exclusively expressed in the proximal tubule of the kidney and have the similar maturation pattern, they may share a common developmental regulatory pathway and could possibly reflect the developmental maturation process of proximal tubule as well.

Genetic polymorphisms of OATs

Genetic polymorphisms of OATs are important to drug disposition of individual person, which are also critical information for personalized therapy in the future. Due to the coding region polymorphisms, different people will show variant drug responses, sometimes-unexpected toxicity or reduced efficacy. Transporters in liver, kidney, intestine and brain are the greatest source of variability, which will result in different drug disposition profiles. Here we focused on those variants occurred in the coding regions of OATs. Some of these variants are synonymous, which will not cause the amino acid changes; the others causing amino acid changes are nonsynonymous. Recently there were several studies have put efforts on characterizing OATs coding region polymorphisms and also exploring the relationships between these OAT genetic variants and transporter functions. Table 5 listed all the nonsynonymous variants of OATs, which have been identified to date, and also their functional analysis, potential mechanisms causing the functional changes.

As to hOAT1, the two single nucleotide polymorphisms (SNPs), R50H and K525I were found to only exist in the samples from African origin (62). Interestingly, the variant K525I has the unchanged affinity for PAH and those nucleoside phosphonate analogs (adefovir, cidofovir and tenofovir); while R50H had lower affinity for those substrates.

In the study of hOAT3, it was found out that among all the screened population, hOAT3 variants are in low frequency (63). There were 10 distinct coding region polymorphisms identified, among which R149S, Q239Stop and I260R have lost the transporter function to uptake ES and cimetidine (CIM) dramatically. The variant I305F has lower uptake of ES but a slightly higher uptake of CIM, which was due to the changed substrate affinities for ES and CIM compared with wild type hOAT3, while the variant R277W had lower uptake for ES and slightly lower uptake of CIM. In another study of hOAT3 polymorphisms, two more genetic variants of hOAT3 have been observed, one is synonymous and the other one is nonsynonymous A389V (64). The functional data showed that there were no significant differences in the mean Clr and CLsec of pravastatin of A389V compared with wild type.

Several genetic variants of hOAT4 have been identified, but no functional data have been characterized (65).

There were many coding region nonsynonymous polymorphisms of URAT1 have been reported (39, 66-68). The mutants, W258Stop, T217M and E298D, have significantly lost transporter functions and were involved with idiopathic renal hypouricemia with exercise-induced acute renal failure and chronic renal

dysfunction (39). The patients with renal hypouricemia have increased renal urate clearance, low levels of serum urate without any underlying renal or systemic diseases. The population has been identified to have high frequency of this disease is Japanese and non-Ashkenzai Jews (69-73). And the mutant W258Stop was reported to be the predominant mutant of URAT1 in Japanese and Korean renal hypouricemia patients (66, 67). According to the immunostaining analysis, the mutant, W258Stop resulted in a truncated protein, which can not properly target to cell membrane, while T217M and E298D mutants have not altered the cell surface expression (39). In another study, seven more mutants of URAT1 have been screened out from the patients with renal hypouricemia (67). The mutant Q297Stop, which was detected in a patient with acute renal failure (ARF), could also result in a truncated protein. The mutants R90H, V138M and frame shift (IVS2+ 1G→A) almost had no urate uptake, while the mutants G164S, Q382L and M430T had significantly reduced urate uptake. From immunocytochemical analysis of these mutants expressing in oocytes, it was found out that the mutants R90H, V138M, G164S and Q382L had no impaired cell surface expression, while the mutants M430T and IVS2+1G→A had very weak cell surface labeling.

Although there were already a lot of coding region polymorphisms of OATs have been identified, but there was few functional analysis about them. To elucidate the function of these coding region polymorphisms of OATs will be very helpful to develop the personalized therapy in the future. And the statistical analysis of these variants will give us some clues on the human evolution with different genetic background, living styles and also nutritional conditions. And this

information will lead us to more considerations, when designing clinical drugs, evaluating the side effects and choosing proper drug delivery system for these drugs later on.

Pharmacokinetics and pharmacodynamics of OATs

The pharmacokinetics of a drug is decided by its physicochemical properties and its interaction with the transporters and metabolic enzymes involved. The difference in the distribution of transporters could be one of the determinants of the elimination route of drugs. Since OATs have various expressions in different human organs, the knowledge about OATs can provide us a molecular basis of the pharmacokinetics of drugs and is useful for the development of drugs with a favorable distribution in the body.

Since the multispecific substrate recognition of OATs, drug-drug interaction can contribute to be another important aspect to affect drug pharmacokinetics. The coexisting of drugs in the plasma may cause the competition of different drugs for the same transport system and influence the pharmacokinetics of each other. For example, the coadministration of OAT substrates, methotrexate, with NSAIDs, penicillins, or probenecid will cause the severe suppression of bone marrow through inhibition of the tubular secretion of methotrexate (74). Similar OAT-involved drug-drug interactions have also been reported in many other cases, such as affecting the efficacy of diuretic action of diuretics. When diuretics are used together with other inhibitors of renal OATs, it will cause the decrease of tubular secretion of diuretics and thus diminish diuretic effects of the drugs (75).

The knowledge to OATs is also important for drug pharmacodynamics. When applied with uricosuric drugs, such as probenecid, benzbromarone, it can get *cis*-inhibitory effect on URAT1-mediated transport of urate and enhance urate secretion; when applied with anti-uricosuric drugs, such as pyrazinoic acid, it can get *trans*-stimulatory effect on the URAT1-mediated transport of urate and inhibits urate excretion (39). This information indicated us that URAT1 could be used as a target for drug design of modulating the serum level of urate in the body.

Physiological and pathological roles of OATs

OATs are responsible for the renal excretion of anionic drugs, which share largely overlapping substrate specificities. These intracellular accumulated organic anions are also the driving force for the urate/organic anion exchanger, URAT1, in the apical surface of proximal tubular cells. hOAT2 is also in charge of the hepatic reabsorption of organic anions, while hOAT1 and hOAT3 were identified to express in choroids plexus, where they absorb organic anions from cerebrospinal fluid. hOAT4 mediates both the reabsorption of organic anions in renal proximal tubule and the excretion of toxic anions from fetus to maternal circulation in the placenta. Since mOAT5 and rOAT5 were also confirmed to express at the apical surface of renal proximal tubular cells, they may act as a renal reabsorption pathway. The newly cloned mOAT6 was found to express mainly in olfactory mucosa and weakly in testis, but not in kidney or brain, which suggested us the possibility that mOAT6 played an important role in the transport system of the nose, as well as that of the testis. URAT1 is localized in the apical

membrane of renal proximal tubular cells, in which it is involved in the renal reabsorption of urate in order to maintain the uric acid homeostasis in blood.

The data collected from the knockout mice model of OAT1 (*Oat1*$^{-/-}$ mice) showed us that after disturbed the expression of OAT1, the renal excretion of organic anions was impaired significantly as well as that of OAT3 knockout mice model (*Oat3*$^{-/-}$ mice) (33, 50). In addition, in the *Oat3*$^{-/-}$ mice, the excretion and absorption of choroid plexus were also disrupted (31). These suggested us the important roles of OAT1 and OAT3 in renal excretion and detoxification, and also in the disposition of organic anions in central nervous system (CNS). Furthermore, it was reported that OAT3 were missing in the bone of osteosclerosis mice compared with that of wild type littermates, which suggested the possibility that OAT3 may affect the calcification of bone by an unknown mechanism (7). Recently it was discovered that rOAT1 and rOAT3 were dramatically down-regulated in the obstructed kidneys of the bilateral ureteral obstruction (BUO) rats compared with the sham-operated rats (76), which raised the possibility that OAT1 and OAT3 were involved in the BUO disease.

It was known that OAT2 was mainly in charge of the hepatic detoxicification; so the impaired expression of OAT2 may cause the hepatotoxicity of anionic toxins or xenobiotics. Defective hOAT4 expression may lead to the renal reabsorption failure and fetal intoxication, since hOAT4 is one of the most critical transporters expressed in human placenta.

Some studies showed that URAT1 was involved with the hereditary disease, renal hypouricemia (39, 67, 68, 77-79). This disease is highly occurred in

Japanese and non-Ashkenazi Jews (69). Patients with this disease have low serum urate levels. They have no renal or systemic diseases except for the development of nephrolithiasis or exercise-induced acute renal failure. People found out that some patients with this disease have defects in URAT1 (67, 68, 79). The most frequently found mutation W258Stop of URAT1 resulted in a premature truncated protein, which completely lost the transporter function due to the deficiency in targeting to cell membrane (67). This is the first report on the genetic diseases related with OATs.

Structure-function relationship and regulation of OATs

Structure-function relationship of OATs

According to the initial sequence analysis prediction, OATs were predicted to share some common features on their secondary structures (Fig 3): 1) having putative 12 transmembrane domains (TMs); 2) their carboxyl- and amino-terminus are intracellular; 3) there are multiple glycosylation sites on the first extracellular loop between TM1 and TM2; 4) there are several phosphorylation sites on the intracellular loop between TM6 and TM7 as well as in the carboxyl terminus (5, 80). In order to confirm this topological structure, a lot of experimental data are still needed. The importance to study these structural characters is to explore their roles in maintaining OATs functions. A lot of studies have proved that some structural features of OATs are very critical for properly targeting transporters to the cell membranes (81-83) or maintaining their substrate binding affinities (81, 84, 85).

1. Post-translational modifications

Protein glycosylation have been demonstrated to be very important in maintaining protein functions and protein folding, helping protein properly targeting and also stabilizing protein (86-92). The work of our lab have explored the role of N-glycosylation on OATs and also provided systematical experimental data to testify the structure prediction of OATs.

Initially it was found out that after the treatment of endoglycosidase F to remove all the sugar moieties on the protein, the molecular mass of mOAT1 shifted from ~90KDa to ~55KDa in mouse kidney membrane fraction (Fig 4A) (82). Furthermore, after treated mOAT1 expressing Cos7 cells with tunicmycin (an inhibitor of asparagines-linked glycosylation), transporter function was dramatically affected and mOAT1 could not target to the plasma membrane as efficiently as the one without tunicmycin treatment (Fig 4B) (82). This indicated that mOAT1 is N-glycosylated and the glycosylation of mOAT1 is very critical to maintain transporter function in affecting mOAT1 targeting to cell membranes.

After that, human and mouse OAT1 have been studied by disrupting its N-glycosylation using mutagenesis analysis (84). Both in mOAT1 and hOAT1 mutating the conserved putative glycosylation site N39 into glutamine caused completely functional lose without affecting their cell surface expression (Fig 5A and 5B). Interestingly N39 has been identified to be glycosylated in hOAT1 while none glycosylated in mOAT1 (Fig 5B). This suggested us that the mOAT1 and hOAT1 functional changes were not due to deglycosylation of N39 *per se*, but possibly because of affecting substrate binding/recognition. And there was no

significant functional change after disrupting the other putative glycosylation sites individually, but the functions of mOAT1 and hOAT1 could be impaired significantly by replacing all the putative glycosylation sites (Fig 5C), which has been proved to be affecting transporter properly targeting to cell membranes (Fig 5D). These data indicated that it is not essentially required to be glycosylated in each putative glycosylation site of human and mouse OAT1, but the simultaneous glycosylation at these sites played important roles in targeting human and mouse OAT1 to the cell membranes.

B. Phosphorylation of OATs

According to the putative secondary structure of OATs, OATs is predicted to have multiple potential phosphorylation sites in the intracellular loop between transmembrane domains 6 and 7, and in the carboxyl terminus (Fig 3). This suggested that OATs might be functional regulated through phosphorylation.

As reported previously, phosphorylation played critical roles in the regulation of many protein functions (93-97). In order to explore the role of phosphorylation played in regulation of OAT functions, studies have been done in the mouse OAT1 expressed LLC-PK1 cells (98). The phosphorylation level of mOAT1 were increased significantly under the treatment of okadaic acid, a protein phosphatase (PPA/PP2A) inhibitor (Fig 6A), and caused a decreased PAH transport in a dose- and time-dependent manner (Fig 6B and 6C). According to the phosphoamino acid analysis, one or more serine residues of mOAT1 have been phosphorylated after the treatment of okadaic acid (Fig 6D), which indicated that this

phosphorylation on serine residues was involved with the okadaic acid induced down regulation of PAH transport by mOAT1.

C. Cell surface assembly of OATs

There were many data showed that transporters are functional as oligomers, such as the member of the ATP binding cassette (ABC) transporter superfamily, human ABCG2, which is functional as a dimer or higher order oligomer (99); and human serotonin transporter also works as a dimer or possiblely a tetramer with functional interactions between each subunit (100).

But there were no reports about the quaternary structure of OATs before. The work of our lab on hOAT1 oligomerization was the first progress in the study of OAT's structural assembly until now (101). It was found out that hOAT1 expressing LLC-PK1 cells can be chemically cross-linked by BS_3 (a noncleavable, water-soluble and membrane-impermeable cross-linking reagent) the same as by another cross-linking reagent DSS (a noncleavable, membrane permeable cross-linking agent) (Fig 7A). And consistent results from cross-linking the membrane proteins, which was isolated from rat kidney (Fig 7A), suggested that hOAT1 possibly could form oligomers not only in mammalian cell expression system but also in physiological environment. Furthermore, such oligomers could be broken by SDS, but not β-mercaptoethanol.

The hOAT1-c-myc stable transformant of LLC-PK1 cells was used to extract the cell membrane fraction, and the membrane protein was dissolved in 2% PFO, a mild ionic detergent, which can keep the natural oligomerization status at certain concentration (99, 102, 103). This membrane protein lysate was loaded onto gel

filtration chromatography and followed by immunoblot analysis. From the immunoblot, there was a major peak fraction of hOAT1 membrane protein, which has an estimated molecular mass larger than hOAT1 trimer but smaller than the tetramer (Fig 7A). A putative dimer or trimer was observed in cross-linking and immunoblotting experiments under the same condition. A possible reason for this difference may be hOAT1 oligomer can associate with other solubilized membrane proteins in PFO.

In order to confirm hOAT1 can self associate and form oligomer, co-immunoprecipitation was performed in hOAT1-c-myc stablely expressing but transiently transfected with hOAT1-c-Flag or hOAT1-N-Flag LLC-PK1 cells. Myc antibody could successfully pull down the oligomer of hOAT1-c-myc and hOAT1-c-Flag from the cell lysate dissolved in both 0.5% PFO and 0.5-1% Triton X-100, while Flag antibody was used to detect the hOAT1-c-Flag afterwards (Fig 7B). More studies were performed to confirm the hypothesis that hOAT1 can form homooligomer. hOAT1-c-myc expressing LLC-PK1 cells were biotinylated with sulfo-NHS-LC-biotin (labeling the lysine residue of proteins, membrane impermeable biotinylation reagents) and biotin hydrazide (labeling carbohydrates of protein and membrane impermeable biotinylation reagents), followed by immunoprecipitation with anti-myc antibody, then detected on immunoblot by horseradish peroxidase-conjugated streptavidin (specifically binding to biotin) (Fig 7C). Furthermore, hOAT1-c-myc expressing LLC-PK1 cells were metabolically labeled with [^{35}S] methionine and immunoprecipitated with anti-myc antibody, followed by gel electrophoresis and photo-imaging (Fig 7C). In both of these two

experiments, there was no other signal with different molecular mass from hOAT1 monomer can be detected. In additional experiment, cell surface proteins were biotinylated with sulfo-NHS-LC-biotin and then crosslinked with DTSSP (a thio-cleavable cross-linker), followed by immunoprecipitation with anti-myc antibody. After treated with reducing reagent, dithiothreitol and immunoblotting with horseradish peroxidase-conjugated streptavidin, there was still no other proteins with the molecular weight different from hOAT1 itself could be detected (Fig 7C). All these evidences showed us that hOAT1 could self associate and form oligomers when expressed in LLC-PK1 cells.

2. Important molecular domains of OATs

Another important common predicted structure of OATs is that they all have 12 putative transmembrane domains (TMs). TMs are reported to be responsible for the transporter substrate translocation (104), correct membrane targeting, folding, and/or intracellular processing (105) and also maintaining transporter oligomerization structures (106).

As discovered in the study of N-glycosylation in mOAT1 and hOAT1, the mutant N39Q, which is conservatively located in the first TM of both mOAT1 and hOAT1, possiblely impaired transporter function not due to protein glycosylation, but because of affecting substrate binding (84). This indicated the possibility that N39 is part of the substrate binding pocket of OAT1, so further study has been done to explore the importance of this whole TM of hOAT1 (81).

Alanine scanning mutagenesis has been applied to construct mutants of hOAT1 on the 1st TM (amino acid 16-37). Two critical amino acids have been

selected by the measurement of mutant functions: Leu-30 and Thr-36, which completely lost transporter functions. By further mutating Leu-30 into several other amino acids (glycine, valine, isoleucine) with varying sizes of side chains, it was found out that transporter function was increasingly impaired with the progressively smaller side chains at this position (Fig 8A), which is resulted in reducing the transporter cell surface expression (Fig 8B).

Distinctly from Ler-30, Thr-36, another critical amino acid in the 1st TM of hOAT1, turned out to be critical in substrate recognition. When mutating Thr-36 into alanine, serine and cysteine, which are different from threonine by missing the methyl group or the hydroxyl group or both of them, it led to the lose of transporter function significantly without affecting transporter cell surface expression (Fig 8B). This will be a piece of valuable data that contribute to the work on figuring out the hOAT1 substrate-binding pocket later on.

This study was the first molecular evidence to explore the role of TMs played in OATs. Hopefully more studies could be done to identify the significance of other TMs of OATs. All of these data will lead us to a clearer understanding of OATs at molecular level.

3. Critical Amino Acids of OATs

Up to date, there are a lot of critical amino acids have been identified in several OAT members through mutagenesis studies (Table 6). The cysteine residues in mOAT1 are important for transporter trafficking to the plasma membrane (107). The aromatic transmembrane residues of rOAT3 (W334, F335, Y341A, Y342Q, F362S) played essential roles in substrate recognition, as well as the conserved

basic amino acids (R454D and K370A) (108, 109). Below are two of my studies on critical amino acids of OATs to further explore the important roles of these amino acids played on OAT functions.

4. Protein-protein Interaction of OATs

PDZ (PSD-95, *Drosophila* discs-large protein, *Zonula occludens* proteins 1) motif has been reported to be an important protein-protein interaction module. Among all the OATs, hOAT4 and URAT1 have been detected to have PDZ motifs (S/T-X-hydrophobic residue) at their Carboxyl-terminus. The yeast two-hybrid screen data showed that hOAT4 can associate with two multivalent PDZ domain-containing protein PDZK1 and NHERF1, while URAT1 can interact with PDZK1 (110, 111). Surface plasmon resonance analysis, in vitro binding assay, immuno-colocalization and coimmunoprecipitation studies confirmed the associations between hOAT4/URAT1 and these PDZ domain-containing proteins, also revealed us the requirement of the PDZ motifs in hOAT4/URAT1 for these interactions. Transporter functional assay showed that these associations could enhance transporter functions (1.2 fold to 1.4 fold) in HEK293 cells. Further investigation indicated that these functional augments were due to the enhancement of Vmax, which was reflected by the increased surface expression level of hOAT4 or URAT1. This information indicated us that hOAT4 and URAT1 were possibly involved in the PDZ scaffold regulated system in the renal proximal tubules.

Another important protein-protein interaction modulators are caveolins (Cav), which are integral membrane proteins present in the caveolae in the plasma

membrane. Caveolins have three isoforms: Cav-1, Cav-2 and Cav-3, among which Cav-1 and Cav-2 have been detected to express in kidney (112, 113). They have been reported to regulate the functions of many receptors, transporters and enzyme proteins (114-116). It was not clear whether OATs also could be affected by caveolins until Dr. Cha's group reported the association of rOAT3 with Cav-1 and rOAT1 with Cav-2 (112, 113). The co-immunoprecipitation and confocal microscopy immunocytochemistry detection confirmed the association of rOAT3 with Cav-1 and rOAT1 with Cav-2. After silencing the expression of Cav-1 and Cav-2 with antisense oligo deoxynucleotide method, the transporter functions of rOAT3 and rOAT1 were significantly reduced, which revealed us the important roles of caveolins in maintaining transporter functions of OATs.

Regulation of OATs

It was reported by several studies that OATs could be modulated by protein kinases (15, 98, 117-122). The activation of protein kinase C (PKC) could down-regulate rOAT1, hOAT1, mOAT1 and rabbit OAT3 transporter functions, and this functional inhibition could be reversed by pretreatment of PKC inhibitors (15, 98, 117, 120-122).

In 2000, Dr. You's group identified that in the mouse OAT1 expressed LLC-PK1 cells, the phosphorylation level of mOAT1 were increased significantly under the treatment of okadaic acid (Fig 6A), and caused a decreased PAH transport in a dose- and time-dependent manner (Fig 6B and 6C) (98). According to the phosphoamino acid analysis, one or more serine residues of mOAT1 have

been phosphorylated after the treatment of okadaic acid (Fig 6D), which indicated that this phosphorylation on serine residues was involved with the okadaic acid induced down regulation of PAH transport by mOAT1.

Although there are several phosphorylation sites on OATs, it was discovered that the PKC-induced hOAT1 down-regulation is not because of phosphorylation but due to the carrier retrieval from the cell membrane into the intracellular compartment (121). Whether OATs share the same regulatory pathways need to be further investigated.

OATs also could be regulated by epidermal growth factor (EGF) via mitogen-activated protein kinases (MAPKs) and this activation could lead to an up-regulation of organic anion transport of rabbit OAT3 and hOAT1 (15, 118, 119). And following the activation of EGF, there were the activations of ERK1/2, phospholipase A_2, MAPKs, generation of prostaglandin (PGE_2), adenylate cyclase and protein kinase A (PKA), so PGE_2 and PKA could also be critical factors in the regulation of OATs. In addition, it was reported that phosphatidylinositol 3-kinase (PI3K) acted upstream and tyrosine kinase acted downstream of this pathway (123). And the inhibition of cyclooxygenase 1 (COX 1) could down-regulate the transport function of OAT3 in rabbit proximal tubular S2 segment (15). All these regulatory factors could be considered as targets for physiological regulation of OATs *in vivo.*

Summary

Organic anion transporters (OATs) are a family of transporters, which played important roles in drug disposition of human body. Lots of the substrates of OATs

are widely used drugs, which made them clinically significant. Because of their wide distribution and broad substrate spectra, they are at critical positions in pharmacokinetics and pharmacodynamics as well.

The present data on its structure-function relationship helped to present us a more and more clear topological structure of OATs. The elucidating the secondary structure of OATs is greatly helpful to resolve the crystal structure of OATs finally, to explore the principle, which caused the substrate specificity of OATs, and also can assist us to find more targets to regulate OATs under physiological conditions in order to get better clinical efficacies. And the discoveries of new OATs expanded the picture of OAT family and gave us more targets for the development of clinical drugs.

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Endogenous organic anions	
Cyclic nucleotides	cAMP, cGMP
Dicarboxylates	a-ketoglutarate, glutarate, succinate
Neurotransmitter metabolites	4-hydroxy-3-methoxymandelic acid, 3,4-dihydroxyphenylacetic acid
Others	urate, folate, octanoate
Exogenous organic anions - drugs	
Antibiotics	penicillin G, carbenicillin, amoxicillin, piperacillin, cloxacillin, nafcillin, cephaloridine, cefadroxil
Anti-viral drugs	azidothymidine, acyclovir, amantadine
NSAIDs	salicylate, acetylsalicylate, indomethacin, antipyrine, benzydamine, paracetamol, diclofenac
Diuretics	furosemide, bumetanide, ethacrynic acid, acetazolamide, benthiazide
ACE inhibitors	captopril, enalapril, imidapril, delapril, benazapril, quinapril, ramipril
ATII antagonists	telmisartan, candesartan, valsartan, losartan
Anti-neoplastics	methotrexate, chlorambucil, 6-MP, thioguanine, dacarbazine, azathioprine, aclarubicin
Anti- epileptics	valproate
Exogenous organic anions – environmental chemicals	

Mycotoxins Conjugated substances	ochratoxin A, ochratoxin B, citreoviridin, citrinin, zearalenol, fumonisin B1
Sulphate conjugates	estrone-S, p-nitrophenyl-S, 4-methylumbelliferyl-S, minoxidil-S, a-naphtyl-S
Cysteine conjugates	S-benzyl-cys, CTFC, DCVC, N-acetyl-S-farnesyl-cys
Glucuronide conjugates	b-estradiol 17-G, p-nitrophenyl-G, 4-methylumbelliferyl-G
Glycine conjugates	PAH, o-hydroxyhippurate

Table 1 Anionic substances interacting with or transported by OAT

Transporter Name	Species	Accession Numbers	Tissue Localization	Cellular Localization	References
hOAT1	Human	AB009697/ AF057039/ AF104038/ AF097490/ AF124373	Kidney	BM	(20, 22-24, 117, 124, 125)
			Skeletal muscle	ND	
			Brain	ND	
			Placenta	ND	
rOAT1	Rat	AF008221/ AB004559	Kidney	BM	(16, 17, 126
			Brain	ND	
mOAT1	Mouse	U52842	Kidney	BM	(10, 127)
			Brain	ND	
			Olfactory mucosa	ND	
fOAT1	Winter Flounder	Z97028	Kidney	ND	(18)
pOAT1	Pig	NM_0010012(1	Kidney	ND	(21)
rbOAT1	Rabbit	AJ242871	Kidney	ND	(19)
CeOAT1	C. *elegans*	AF152095			(128)
mkOAT1	Monkey	AB182992	Kidney	ND	(129)
hOAT2	Human	AF097518/ AF210455/ AY050498	Kidney	BM	(25, 130)
			Liver	SM	
mOAT2	Mouse	AB069965/ NM_144856	Liver	ND	(27)
			Kidney	ND	
rOAT2	Rat	L27651	Liver	ND	(12, 28, 131
			Kidney	ND	
hOAT3	Human	AF097491	Kidney	BM	(23, 30, 36, 124, 125)
			Brain	LM	
			Skeletal muscle	ND	
			Adrenal cortex	ND	
rOAT3	Rat	AB017446	Liver	ND	(9, 131)
			Kidney	ND	
			Lung	ND	
			Brain	AM	
			Eye	ND	

mOAT3	Mouse	AF078869	Kidney	ND	(7)
			Endochondral bone	ND	
			Brain	AM	
mkOAT3	Monkey	AB182993	Kidney	ND	(129)
hOAT4	Human	AB026116	Placenta	BM	(8, 34, 36)
			Kidney	AM	
			Adrenal cortex	ND	
hOAT5	Human	AA705512	Liver	ND	(25)
mOAT5	Mouse	NM_144785	Kidney	AM	(13, 38)
rOAT5	Rat	AB051836	Kidney	AM	(37)
mOAT6	Mouse	NM_198650	Olfactory mucosa	ND	(11)
			Testis	ND	
hURAT1	Human	AB071863	Kidney	AM	(39)
mURAT1	Mouse	AB005451	Kidney	AM	(40)

Table 2. Tissue and cellular distribution of OATs.

BM: Basolateral Membrane

AM: Apical Membrane

SM: Sinusoidal Membrane of hepatocytes

LM: Luminal Membrane of Choroid Plexus

ND: Not Determined

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	Species	Substrates	Reference
OAT1	rat	PAH, dicarboxylates, PGE2,urate, various anionic drugs, OTA, etc	(16, 17)
	human	PAH, dicarboxylates, etc.	(20, 22, 24, 117)
	mouse	PAH, dicarboxylates, etc	(10)
	Winter flounder	PAH, dicarboxylates, diuretics, etc	(18)
OAT2	rat	dicarboxylates, salicylate, PGE2, etc	(12, 28)
	human	$PGF_{2\alpha}$, probenecid, etc	(130)
OAT3	rat	estrone sulphate, PAH, cimetidine, OTA, etc	(9)
	human	estrone sulphate, PAH, cimetidine, OTA, etc	(23)
OAT4	human	estrone sulphate, DHEA sulphate, OTA, etc	(8)
OAT5	mouse	Estrone sulphate, DHEA, OTA, nitrophenyl sulfate, methylumbelliferyl sulfate, etc	(13, 38)
	rat	Estrone sulphate, DHEA, OTA, succinate, etc	(13, 37)
OAT6	mouse	Estrone sulfate, 2,4-dichlorophenoxyacetate, OTA, salicylate, penicillin G, PAH, and urate, etc	(46)
URAT1	mouse	Urate, probenecid, benzbromarone, lactate, etc	(40)
	Human	Urate, probenecid, aromatic monocarboxylates, aliphatic monocarboxylates, nonsteroidal anti-inflammatory drugs, and diuretic drugs, etc	(39)

Table 3. Substrate specificity of OATs.

OTA: ochratoxin A

PAH: Para-aminohippuric acid

PGE2: prostaglandin E_2

DHEA: dehydroepiandrosterone sulfate

$PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$

ND: not determined

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Transporter Name	Transporter Mode
hOAT1	OA/DC exchanger
hOAT2	Facilitated diffusion?
rOAT3	OA/DC exchanger
hOAT4	OA/DC exchanger
rOAT5	OA/DC exchanger
mOAT6	OA/DC exchanger
hURAT1	Urate/OA exchanger

Table 4. The transporter modes of OATs

OA: organic anions

DC: dicarboxylate

Gene Name	Coding Region Variants	Functional Analysis	Kinetical Data and Protein Cells Surface Expression Analysis	Reference
hOAT1	R50H	Cidofovir ↑ Adefovir ↑ Tenofovir ↑ PAH —	Decreased K_m for cidofovir, adefovir and tenofovir, unchanged K_m for PAH	1
	K525I	Cidofovir — Adefovir — Tenofovir — PAH —	Unchanged K_m for cidofovir, adefovir, tenofovir and PAH	1
	L7P	N/A		4
hOAT2	T110I	N/A		4
	V192I	N/A		4
	G507D	N/A		4
hOAT3	F129L	ES, CIM —		2
	R149S	ES, CIM no uptake		2
	Q239Stop	ES, CIM no uptake		2
	I260R	ES, CIM no uptake		2
	R277W	ES ↓ , CIM ↓ (not significant)		2
	V281A	ES, CIM —		2
	I305F	ES ↓, CIM ↑ (slightly)	Increased K_m for ES (not significant) Decreased K_m for CIM	2
	A310V	ES, CIM —		2
	A399S	ES, CIM —		2
	V448I	ES, CIM —	Decreased K_m for CIM, Decreased V_{max} for CIM	2
	A389V	CL_r and CL_{sec} of pravastatin —		3
	I175V	N/A		4
hOAT4	V13M	N/A		4
	R48Y	N/A		4
	T62R	N/A		4
	V155M	N/A		4

	A244V	N/A		4
	E278K	N/A		4
	V399M	N/A		4
	T392I	N/A		4
hURAT1	W258Stop	No urate uptake	Impaired to target to cell membrane	5,6,7,8
	T217M	No urate uptake	Unimpaired cell membrane expression	5,6
	E298D	No urate uptake	Unimpaired cell membrane expression	5,6
	Q297Stop	N/A		7
	R90H	No urate uptake	Unimpaired cell membrane expression	7,8
	V138M	No urate uptake	Unimpaired cell membrane expression	7
	G164S	Reduced urate uptake	Unimpaired cell membrane expression	7
	Q382L	Reduced urate uptake	Unimpaired cell membrane expression	7
	M430T	Reduced urate uptake	Impaired to target to cell membrane	7
	Frame shift (IVS2+ 1G→A)	No urate uptake	Impaired to target to cell membrane	7
	R477H	N/A		8

Table 5. Nonsynonymous coding region polymorphisms of human OATs.

↑: increase; —: no change; ↓: decrease.

PAH: Para-aminohippurate

ES: Estrone Sulfate

CIM: Cimetidine

N/A: data not available

$CL_{r:}$ Renal clearance

$CL_{sec:}$ clearance by renal secretion

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OAT isoforms	Mutants	Functional consequence due to the mutation	Topological Localization	Reference
hOAT1	N39Q	Loss of transporting organic anion PAH possibly due to affecting the substrate binding/recognition	EL I	(84)
hOAT1	N39/56/92/ 97Q	Loss of transporting of organic anion PAH due to the impairment of surface expression	EL I	(84)
hOAT1	N39/56/92/ 97/113Q	Loss of transporting organic anion PAH due to the loss of surface expression	EL I	(84)
hOAT1	L30A	Loss of transporting organic anion PAH due to the loss of surface expression	TM 1	(81)
hOAT1	L30G	Loss of transporting organic anion PAH due to the loss of surface expression	TM 1	(81)
hOAT1	L30V	Reduced transport of organic anio PAH due to the reduced surface expression but increased binding affinity for PAH	TM 1	(81)
hOAT1	L30I	Reduced transport of organic anio PAH due to the reduced surface expression but increased binding affinity for PAH	TM 1	(81)
hOAT1	T36A	Loss of transporting organic anion PAH possibly due to changing the substrate recognition	TM 1	(81)
hOAT1	T36S	Loss of transporting organic anion PAH possibly due to changing the substrate recognition	TM 1	(81)
hOAT1	T36C	Loss of transporting organic anion PAH possibly due to changing the substrate recognition	TM 1	(81)
mOAT1	N39Q	Loss of transporting organic anion PAH possibly due to affecting the	EL I	(84)

		substrate binding/recognition		
mOAT1	N56/86/91/ 107Q	Reduced transport of organic anio PAH due to the impairment of surface expression	EL I	(84)
mOAT1	C49A	Reduced sensitivity to the inhibitio by PCMBS	EL I	(107)
mOAT1	C335/379/ 427/434A	Reduced transport of organic anio PAH due to the reduced surface expression		(107)
mOAT1	C-less	Loss of transporting organic anion PAH due to the loss of surface expression		(107)
fOAT1	K394A	Reduced transport of organic anio PAH possibly due to altering the conformational change rate and affecting dicarboxylate binding an PAH/dicarboxylate exchanging	TM 8	(132)
fOAT1	R478D	Reduced transport of organic anio PAH possibly due to altering the conformational change rate and reduced binding affinity for PAH and affecting dicarboxylate binding and PAH/ dicarboxylate exchanging	TM 11	(132)
fOAT1	H34I	Reduced transport of organic anio PAH possibly due to altering the conformational change rate	TM 1	(132)
rOAT3	R454D	Reduced transport of organic anions PAH, estrone sulfate and ochratoxin A due to affecting binding and translocation of organic anions	TM 11	(108)
rOAT3	R454N	Reduced transport of organic anions PAH, estrone sulfate	TM 11	(108)

		and ochratoxin A due to affecting binding and translocation of organic anions		
rOAT3	K370A	Reduced transport of organic anions PAH, estrone sulfate and ochratoxin A due to affecting transport and recognition of PAH	TM 8	(108)
rOAT3	R454D/ K370A	From transporting organic anions to transporting organic cation MPP^+ due to changing the transporter substrate specificit		(108)
rOAT3	W334A	Reduced transport of both organic anion PAH and organic cation cimetidine due to affecting the translocation of PAH and cimetidin	TM 7	(109)
rOAT3	W334F	Reduced transport of both organic anion PAH and organic cation cimetidine due to affecting the translocation of PAH and cimetidin	TM 7	(109)
rOAT3	F335A	Reduced transport of both organic anion PAH and organic cation cimetidine due to affecting the translocation of PAH and cimetidin	TM 7	(109)
rOAT3	Y341A	Reduced transport of both organic anion PAH and organic cation cimetidine due to affecting the translocation of PAH and cimetidin	TM 7	(109)
rOAT3	Y342Q	Reduced transport of both organic anion PAH and organic cation cimetidine due to affecting the	TM 7	(109)

		translocation of PAH and cimetidin		
rOAT3	Y342F	Reduced transport of organic anio PAH and organic cation cimetidine due to affecting the translocation c PAH and cimetidine	TM 7	(109)
rOAT3	F362S	Reduced transport of both organic anion PAH and organic cation cimetidine due to affecting the translocation of PAH and cimetidin	TM 8	(109)
rOAT3	F362A	Reduced transport of both organic anion PAH and organic cation cimetidine due to affecting the translocation of PAH and cimetidin	TM 8	(109)
hOAT4	G241A	Reduced transport of organic anio estrone sulfate due to the reduced surface expression and decreased binding affinity for estrone sulfate	TM 5	(83)
hOAT4	G241S	Reduced transport of organic anio estrone sulfate due to the reduced surface expression and decreased binding affinity for estrone sulfate	TM 5	(83)
hOAT4	G241V	Loss of transporting organic anion estrone sulfate due to the loss of surface expression	TM 5	(83)
hOAT4	G241L	Loss of transporting organic anion estrone sulfate due to the loss of surface expression	TM 5	(83)
hOAT4	G400A	Reduced transport of organic anio estrone sulfate due to the reduced surface expression and decreased binding	TM 8	(83)

		affinity for estrone sulfate		
hOAT4	G400S	Reduced transport of organic anio estrone sulfate due to the reduced surface expression and decreased binding affinity for estrone sulfate	TM 8	(83)
hOAT4	G400V	Loss of transporting organic anion estrone sulfate due to the loss of surface expression	TM 8	(83)
hOAT4	G400L	Loss of transporting organic anion estrone sulfate due to the loss of surface expression	TM 8	(83)
hOAT4	H47A	Reduced transport of organic anio estrone sulfate due to the reduced surface expression	EL I	(133)
hOAT4	H47/52/83A	Reduced transport of organic anio estrone sulfate due to the reduced surface expression	EL I	(133)
hOAT4	H47/52/83/ 305/469A	Reduced transport of organic anio estrone sulfate due to the reduced surface expression		(133)
hOAT4	H469A	Loss of the transporter sensitivity to DEPC treatment	TM 11	(133)
hOAT4	N39/56/63/ 99Q	Loss of transporting organic anion estrone sulfate due to the loss of surface expression	EL I	(85)

Table 8. Summary of OAT mutants and the impact of these mutations on OAT function

m: mouse, r: rat, h: human, f: flounder

PAH: p-aminohippurate,

PCMBS: p-chloromercuribenzenesulphonate

EL: extracellular loop,

TM: transmembrane domain

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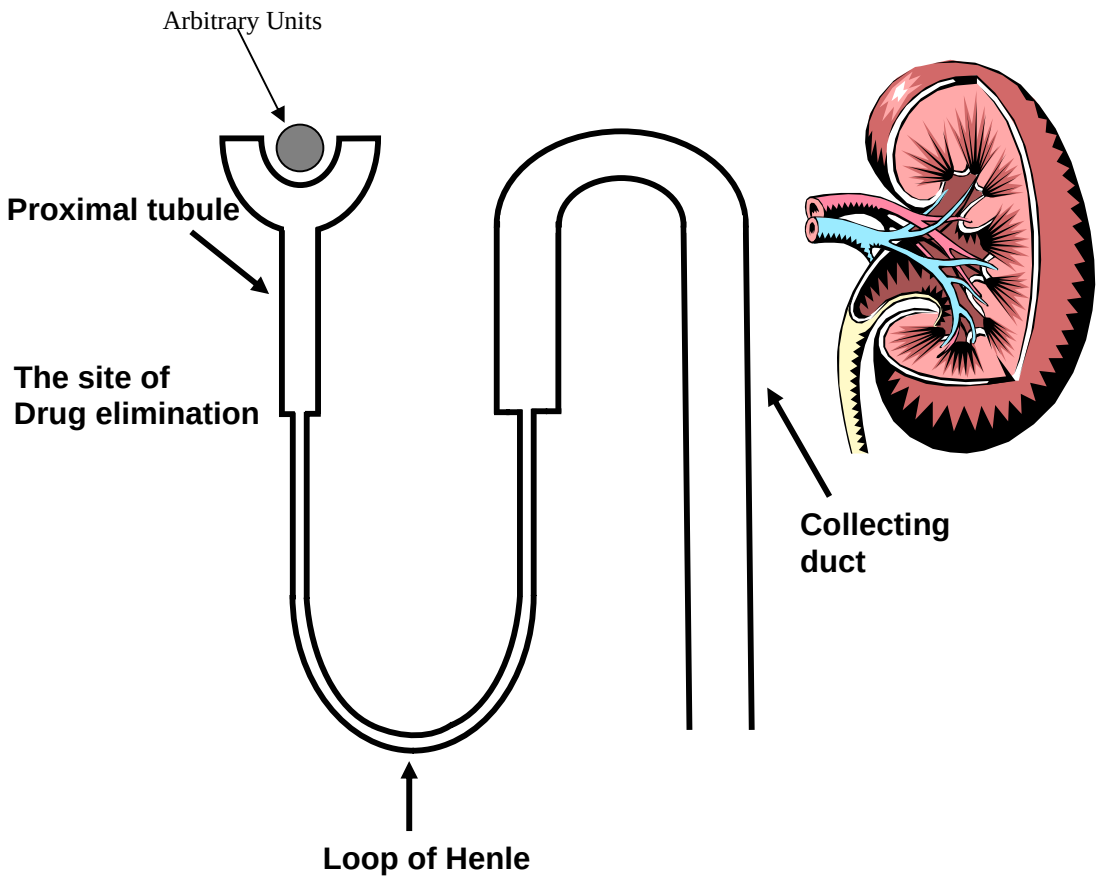

Fig 1. Structure of kidney nephron

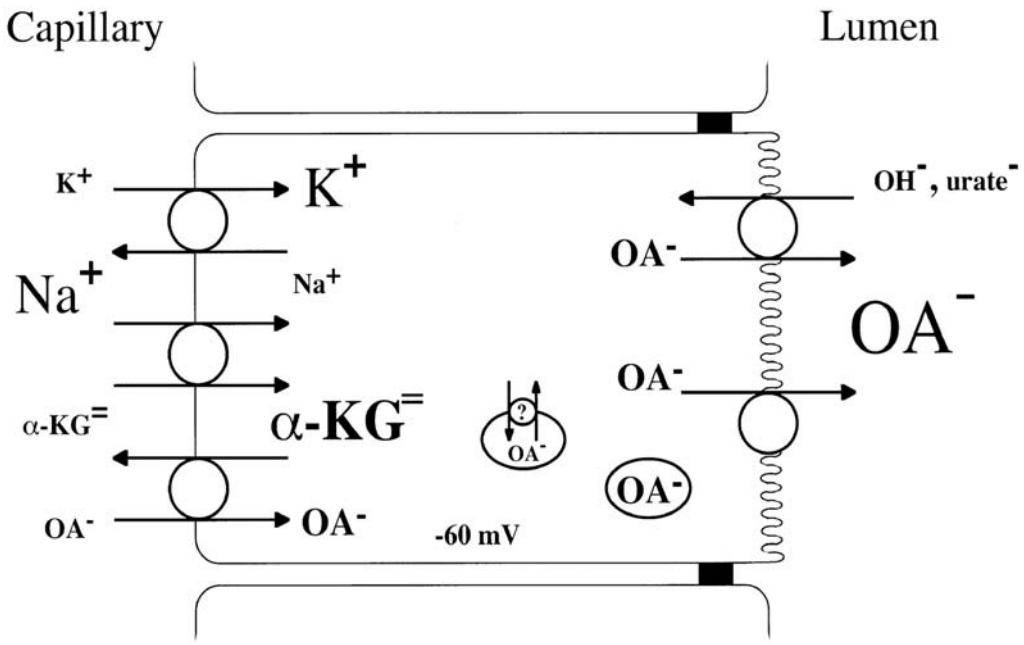

Fig 2. Transport mode of organic anion transporter (OAT).

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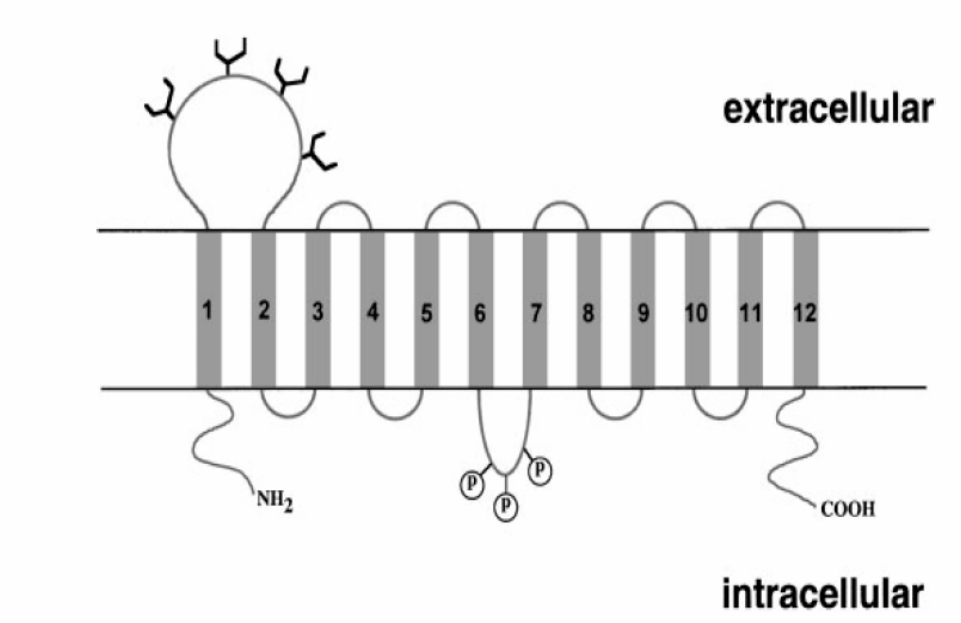

Fig 3. Predicted transmembrane topology of OAT family.

Twelve transmembrane domains are numbered from1-12. Potential glycosylation sites are denoted by tree-like structures. Potential phosphorylation sites are labeled as “P”.

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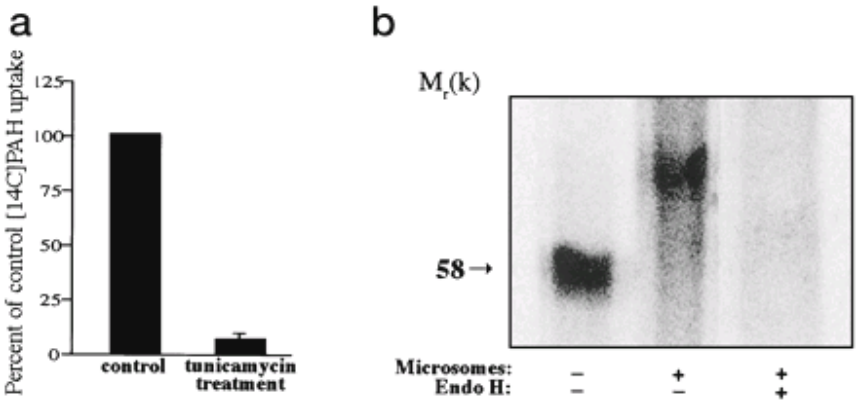

Fig 4A. The effect of glycosylation on the transport function.

a, the effect of tunicamycin on mOAT-mediated PAH transport (20 μM). *b*, *in vitro* translation of mOAT cRNA. The figure shows an autoradiography of an SDS-polyacrylamide gel electrophoresis (7.5%) used to analyze the *in vitro* translation products of mOAT cRNA obtained in the absence (*first lane*) and in the presence (*second lane*) of pancreatic microsomes after centrifugation. The *third lane* shows the product obtained in the presence of microsomes after deglycosylation with endoglycosidase H (*Endo H*).

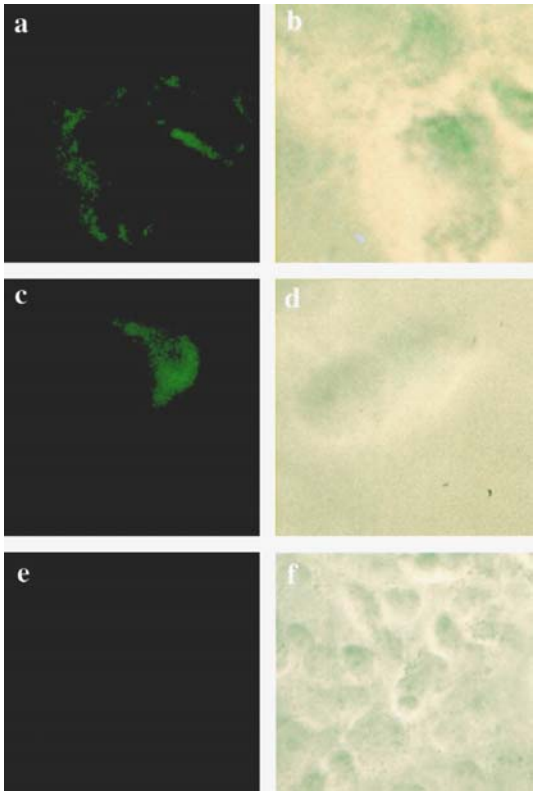

Fig 4B. Immunofluorescence study of the effect of tunicamycin on mOAT expression.

Cells were stained with anti-myc antibody and fluorescein isothiocynate-coupled goat anti-mouse IgG. Specific immunostaining appears as green fluorescence. *a*, *c*, and *e* represent the immnofluorescent microscopy, and *b*, *d*, and *f* represent the corresponding phase contrast microscopy of *a*, *c*, and *e*.

Kuze K, Graves P, Leahy A, Wilson P, Stuhlmann H, You G. Heterologous expression and functional characterization of a mouse renal organic anion transporter in mammalian cells. J Biol Chem. 1999 Jan 15;274(3):1519-24.

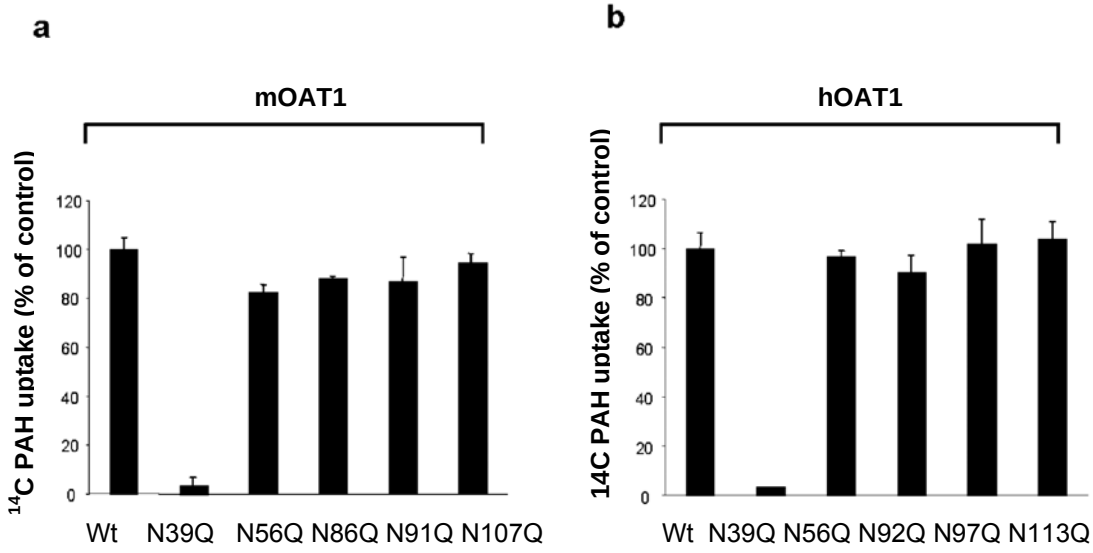

Fig 5A. Functional analysis of the effect of single disruption of putative glycosylation sites.

Mutant transporters were generated by individually replacing asparagine (*N*) with glutamine (*Q*) at each potential glycosylation site of mOAT1 (*a*) and hOAT1 (*b*). The wild type (*Wt*) and mutants were expressed in HeLa cells followed by measuring [^{14}C] PAH uptake (20 mM). The data are presented as the percentage of the control. Values shown are the mean ± S.E. (*n* = 3).

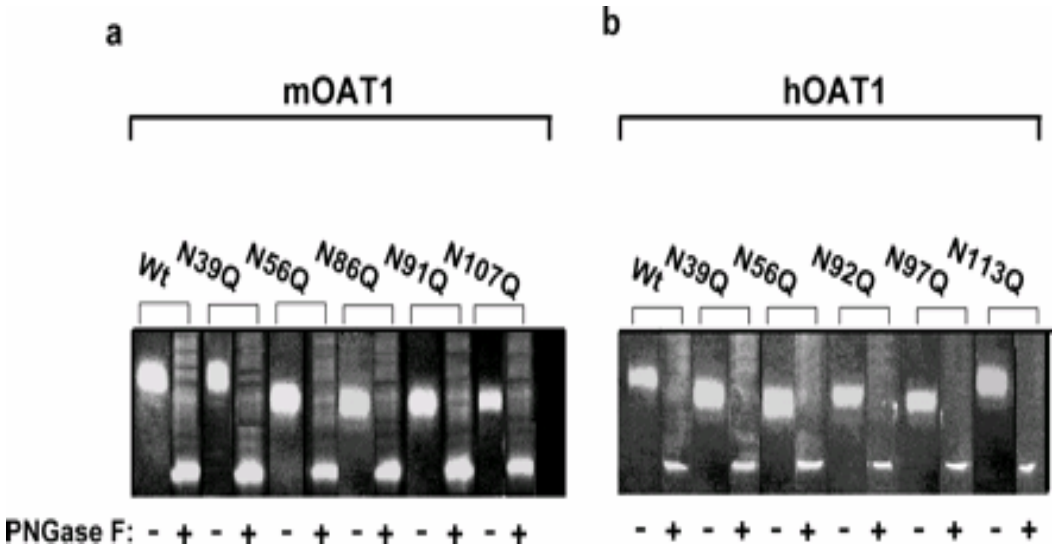

Fig 5B. Western blot analysis of the effect of single disruption of putative glycosylation sites.

Mutant transporters were generated by individually replacing asparagine (*N*) with glutamine (*Q*) at each potential glycosylation site of mOAT1 (*a*) and hOAT1 (*b*). The wild type (*Wt*) and mutants were expressed in HeLa cells. Plasma membrane proteins were isolated via chromatography on streptavidin-agarose after surface biotinylation as described under "Experimental Procedures." The proteins were then treated with (+) or without (-) PNGase F followed by separation on 7.5% SDS-PAGE, transfer onto polyvinylidene difluoride membrane, and probing with anti-*myc* antibody.

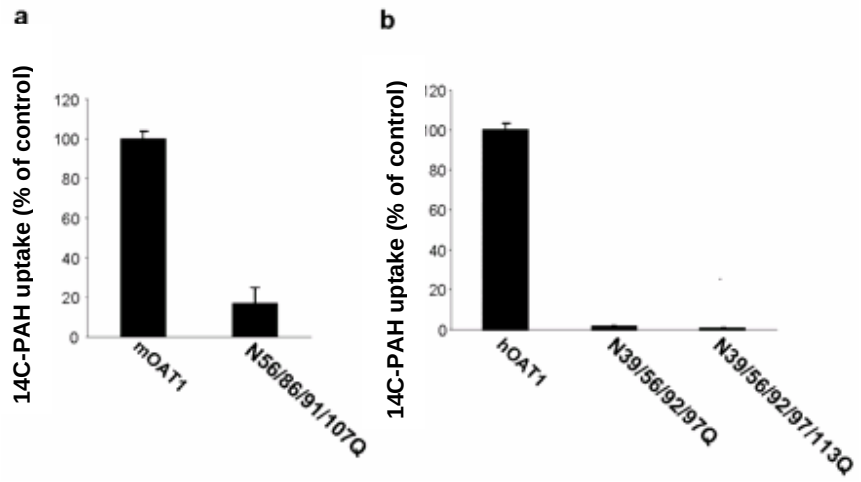

Fig 5C. Functional analysis of the effect of multiple disruptions of putative glycosylation sites.

Mutant transporters were generated by simultaneously replacing all the asparagines (*N*) with glutamine (*Q*) in mOAT1 (*a*) and hOAT1 (*b*). The mutants were expressed in HeLa cells followed by measuring [^{14}C]PAH uptake (20 mM). The data are presented as the percentage of the control. Values are the mean ± S.E. (*n* = 3).

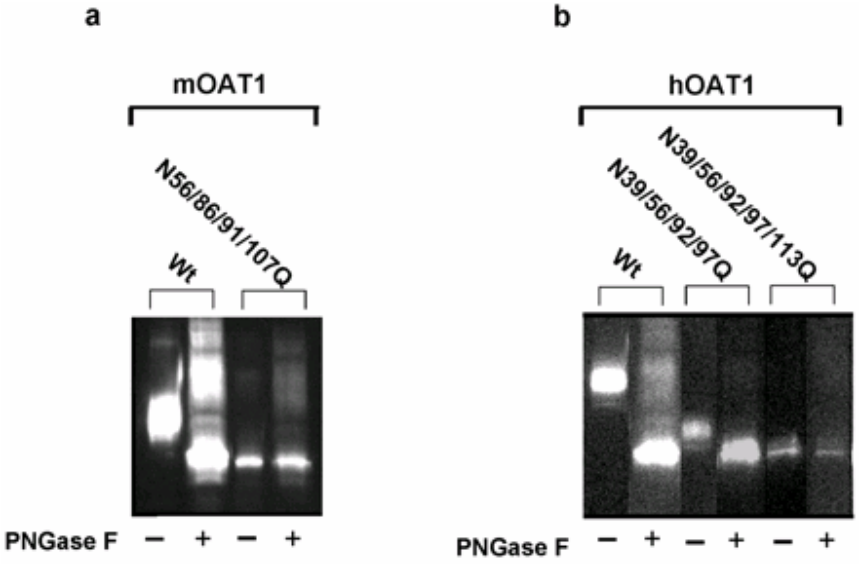

Fig 5D. Western blot analysis of the effect of multiple disruptions of putative glycosylation sites.

Mutant transporters were generated by simultaneously replacing all the asparagines (*N*) with glutamine (*Q*) in mOAT1 (*a*) and hOAT1 (*b*). The wild type (*Wt*) and mutants were expressed in HeLa cells. Plasma membrane proteins were isolated through biotinylation from these cells. The proteins were then treated with (+) or without (-) PNGase F followed by separation on 7.5% SDS-PAGE, transfer onto polyvinylidene difluoride membrane, and probing with anti-*myc* antibody.

Tanaka K, Xu W, Zhou F, You G. Role of glycosylation in the organic anion transporter OAT1. J Biol Chem. 2004 Apr 9;279(15):14961-6.

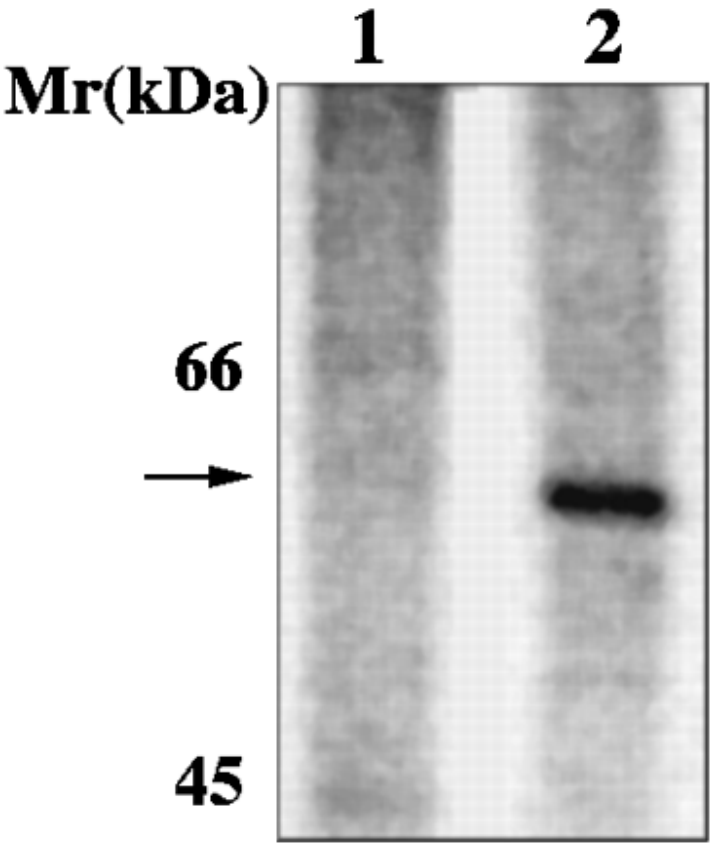

Fig 6A *In vivo* phosphorylation of mOAT in LLC-PK$_1$ cells.

Cells stably transfected with pcDNA3.1(−) (*lane 1*) or pcDNA3.1(−)-mOAT-myc (*lane 2*) were labeled with [^{32}P]orthophosphate and incubated with okadaic acid. Cell lysates were immunoprecipitated with anti-myc antibody, electrophoresed, and autoradiographed. The *arrow* indicates phosphorylated protein.

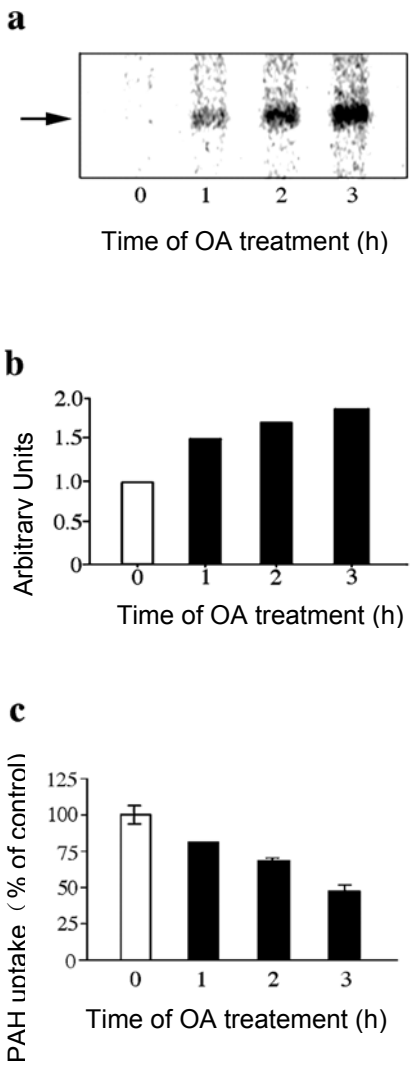

Fig 6B Time course of okadaic acid-modulated phosphorylation of mOAT and PAH uptake.

Transfected LLC-PK_1 cells were labeled with [^{32}P]orthophosphate in phosphate-free DMEM for 1 h at 37 °C and incubated with 1 μM okadaic acid (*OA*) for various times. RIPA extraction, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described under "Experimental Procedures." *a*, representative autoradiogram of labeling results. *b*, quantitation of mOAT labeling. *c*, 1-min basolateral uptake of PAH (20 μM) into the cells treated with 1 μM okadaic acid for indicated time periods. Values are mean ± S.E. (n = 3).

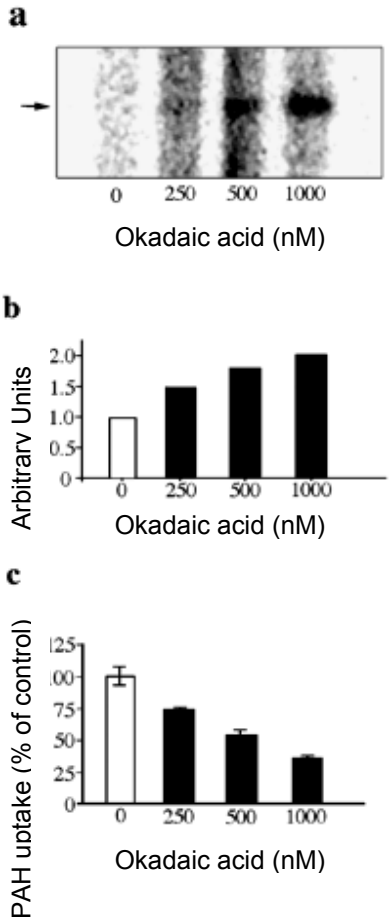

Fig 6C. Dose response of okadaic acid for the phosphorylation of mOAT and PAH uptake.

Transfected LLC-PK_1 cells were labeled with [^{32}P] orthophosphate in phosphate-free DMEM for 1 h at 37 °C and incubated with the indicated concentrations of okadaic acid for 3 h. RIPA extraction, immunoprecipitation, SDS-PAGE, and autoradiography were performed as described under "Experimental Procedures." *a*, representative autoradiogram of labeling results. *b*, quantitation of mOAT labeling. *c*, 1-min basolateral uptake of PAH (20 μM) into the cells treated with okadaic acid at the indicated concentrations for 3 h. Values are mean ± S.E. (*n* = 3).

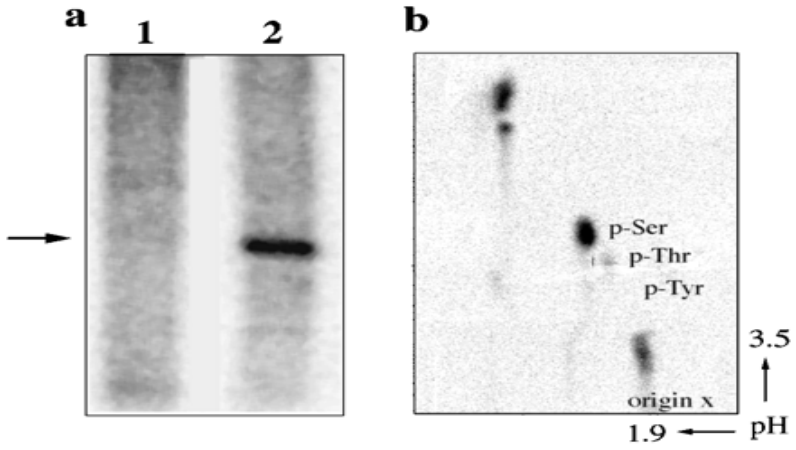

Fig 6D. Phosphoamino acid analysis of mOAT.

a, PhosphorImager analysis of [^{32}P]phospho-mOAT. LLC-PK$_1$ cells stably transfected with pcDNA3.1(−) alone (*lane 1*) and mOAT-myc-pcDNA3.1(−) (*lane 2*) were incubated with okadaic acid, metabolically labeled with ^{32}P, and immunoprecipitated with anti-myc antibody. After SDS-PAGE, the phosphorylated mOAT was transferred onto PVDF membrane.

b, phosphoamino acid analysis of mOAT. Phosphorylated mOAT from *a* was excised from the PVDF membrane and hydrolyzed in hydrochloric acid, and the resulting phosphoamino acids were separated with thin layer electrophoresis using pH 1.9 for the first dimension and pH 3.5 for the second dimension. Autoradiography shows radiolabeled material co migrating with the phosphoserine (*p-Ser*) standard, but not with phosphothreonine (*p-Thr*) or phosphotyrosine (*p-Tyr*). Phosphopeptides resulting from incomplete hydrolysis are seen as a smear in the second direction. The origin is indicated.

You G, Kuze K, Kohanski RA, Amsler K, Henderson S. Regulation of mOAT-mediated organic anion transport by okadaic acid and protein kinase C in LLC-PK(1) cells. J Biol Chem. 2000 Apr 7;275(14):10278-84

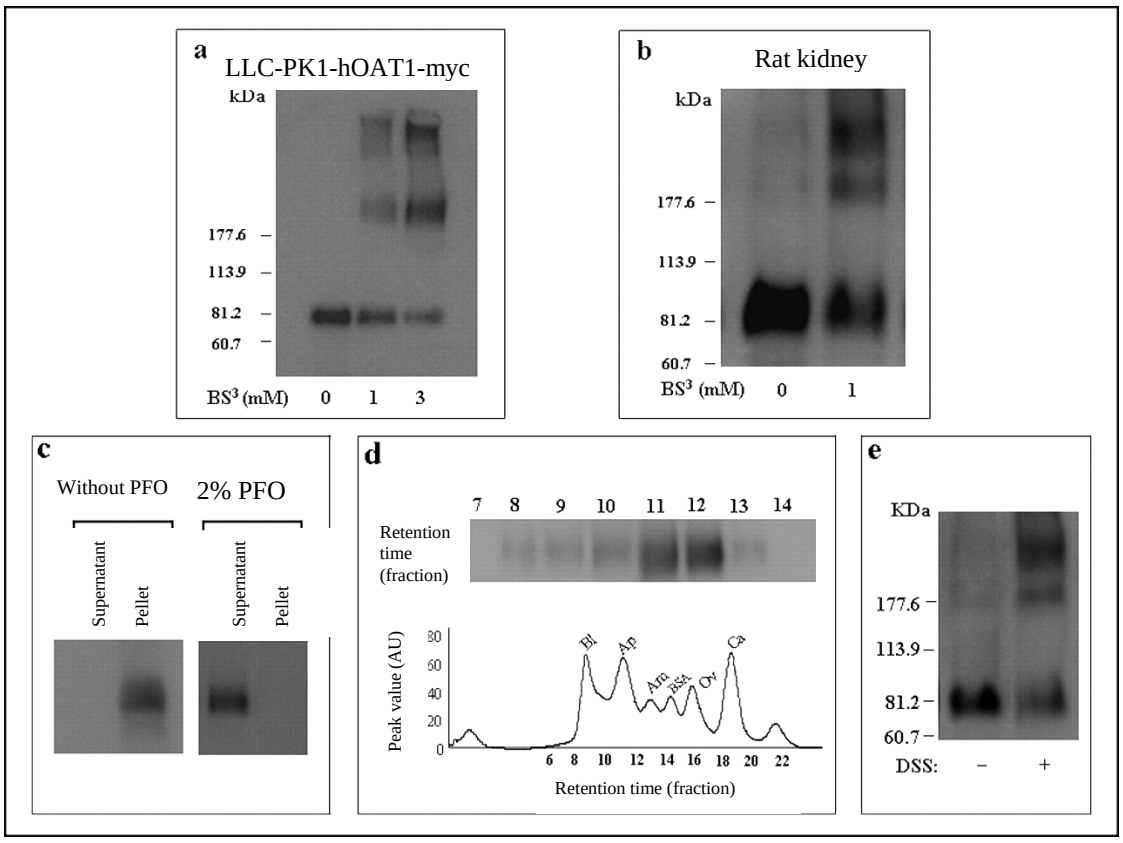

Fig 7A.Oligomerization of hOAT1.

a, cross-linking of membrane proteins from LLC-PK1 cells expressing hOAT1-myc. Total membrane proteins were cross-linked with a cross-linker BS^3. The cross-linked proteins were then subjected to immunoblotting with anti-myc antibody. The apparent molecular masses of hOAT1 oligomers were estimated based on the linear regression of the molecular mass markers used.

b, cross-linking of membrane proteins from rat kidney. Total membrane proteins were cross-linked with BS^3. The cross-linked proteins were then subjected to immunoblotting with anti-rOAT1 antibody (Alpha Diagnostic International, Inc., San Antonio, TX). The apparent molecular masses of rOAT1 oligomers were estimated based on the linear regression of the molecular mass markers used.

c, PFO extraction of hOAT1 from LLC-PK1 cells membranes. Crude membranes from hOAT1-expressing LLC-PK1 cells were treated with/without 2% PFO-containing buffer followed by centrifugation at 11,000 x *g* to separate the

supernatant from the pellet. All supernatant and pellet were subjected to immunoblotting with anti-myc antibody.

d, gel filtration chromatography of hOAT1. *Top panel*, the supernatant from 100 μg of membrane proteins extracted with 2% PFO was separated by gel filtration chromatography. The fractions were collected and precipitated with trichloroacetic acid for SDS-PAGE and immunoblot analysis. *Bottom panel*, retention times of markers blue dextran (*Bl*, 2,000,000, as void volume marker), apoferritin (*Ap*, 443,000), β-amylase (*Am*, 200,000), bovine serum albumin (*BSA*, 66, 000), ovalbumin (*Ov*, 45,000), carbonic anhydrase (*Ca*, 29,000). The apparent molecular masses of hOAT1 were estimated based on the linear regression of the relative retention time of the molecular mass markers. *e*, cross-linking of hOAT1 in detergent PFO. Membrane proteins from hOAT1-myc-expressing cells were solubilized in 2% PFO, followed by cross-linking with 3 mM DSS. The proteins were then analyzed by electrophoresis and immunoblotting with anti-myc antibody.

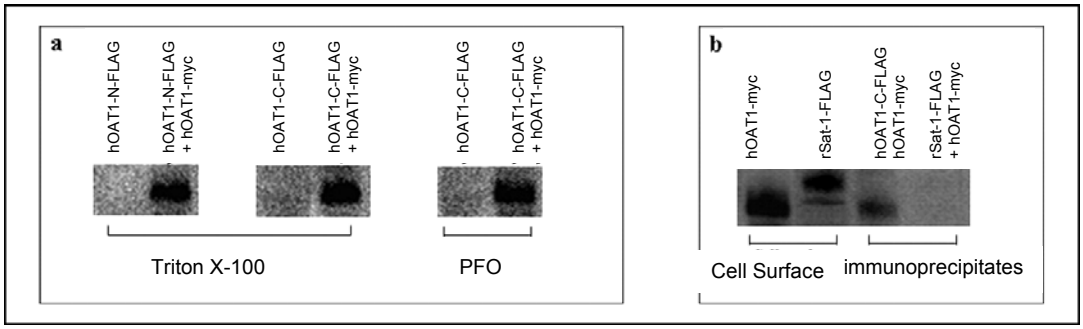

Fig 7B. Association of hOAT1-FLAG with hOAT1-myc.

a, co-immunoprecipitation of hOAT1-FLAG with hOAT1-myc. LLC-PK1 cells co-expressing hOAT1-myc and hOAT1-FLAG were lysed in either 0.5% PFO or 0.5–1% Triton X-100 and subjected to immunoprecipitation with anti-myc antibody, followed by immunoblotting with horseradish peroxidase-conjugated anti-FLAG antibody. *hOAT1-N-FLAG*, FLAG tag was added to the amino terminus of hOAT1; *hOAT1-C-FLAG*, FLAG tag was added to the carboxyl terminus of hOAT1. *b*, co-immunoprecipitation of rSat-1-FLAG with hOAT1-myc. Cell surface expression of rSat-1-FLAG and hOAT1-myc were determined by biotinylation with NHS-SS-biotin, followed by SDS-PAGE and immunoblotting. Association of rSat-1-FLAG with hOAT1-myc was determined in cells co-expressing rSat-1-FLAG and hOAT1-myc through immunoprecipitation with anti-myc antibody, followed by immunoblotting with horseradish peroxidase-conjugated anti-FLAG antibody.

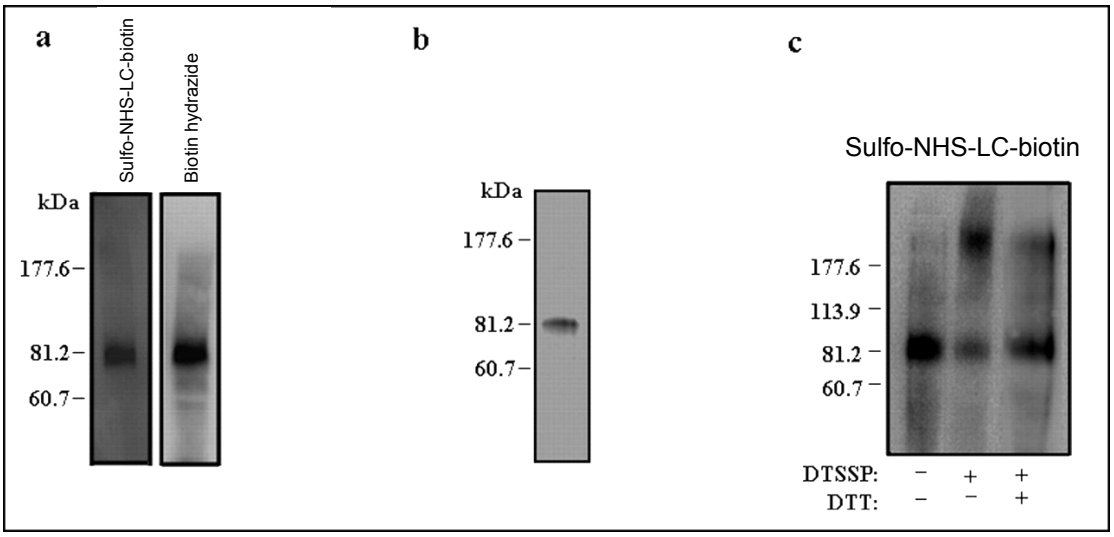

Fig 7C. Analysis of hOAT1 oligomers by cell surface biotinylation and metabolic labeling.

a, cell surface biotinylation. hOAT1-myc-expressing LLC-PK1 cells were biotinylated with lysine-specific sulfo-NHS-LC-biotin or carbohydrate-specific biotin hydrazide. Labeled cells were lysed, immunoprecipitated with anti-myc antibody, and subjected to immunoblotting with horseradish peroxidase-conjugated streptavidin.

b, metabolic labeling. hOAT1-myc-expressing LLC-PK1 cells were metabolically labeled with [^{35}S]methionine and then immunoprecipitated with anti-myc antibody followed by separation with SDS-PAGE for autoradiography.

c, cell surface biotinylation followed by cross-linking with a thiocleaveable cross-linker DTSSP. hOAT1-myc-expressing LLC-PK1 cells were biotinylated with lysine-specific sulfo-NHS-LC-biotin and then lysed. Labeled proteins were cross-linked with 5 mM DTSSP, and immunoprecipitated with anti-myc antibody. The immunoprecipitates were treated with 10 mM dithiothreitol and subjected to immunoblotting with horseradish peroxidase-conjugated streptavidin.

Hong M, Xu W, Yoshida T, Tanaka K, Wolff DJ, Zhou F, Inouye M, You G. Human organic anion transporter hOAT1 forms homooligomers. J Biol Chem. 2005 Sep 16;280(37):32285-90.

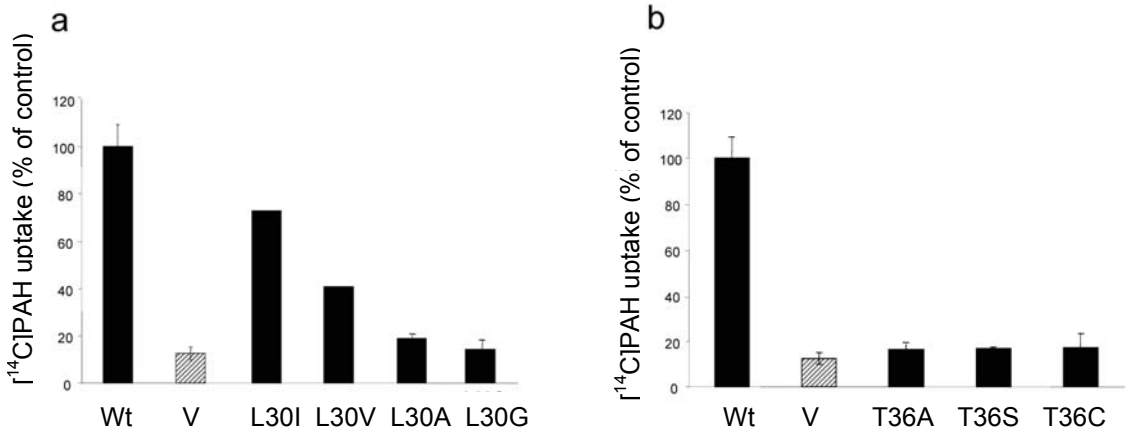

Fig 8A. The effect of mutations at Leu-30 and Thr-36 on hOAT1 function. *a,* PAH uptake in cells expressing mutants of Leu-30. PAH uptake was measured in cells expressing hOAT1 wild-type (*Wt*), pcDNA vector (*V*), L30G, L30A, L30V, and L30I. *b*, PAH uptake in cells expressing mutants of Thr-36. PAH uptake was measured in cells expressing hOAT1 wild-type, pcDNA vector, T36A, T36S, and T36C. The results shown are means ± S.E. (*n* = 3).

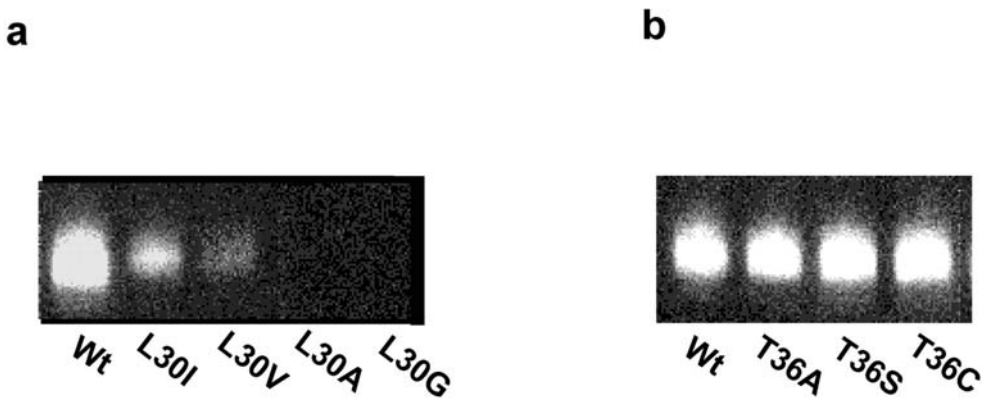

Fig 8B. Surface expression of hOAT1 wild type and its mutants.
Cells expressing hOAT1 wild-type (*Wt*) and its mutants (L30G, L30A, L30V, and L30I in *a*, and T36A, T36S, and T36C in *b*) were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, and visualized by immunoblot analysis using anti-myc antibody.

Hong M, Zhou F, You G. Critical amino acid residues in transmembrane domain 1 of the human organic anion transporter hOAT1. J Biol Chem. 2004 Jul 23;279(30):31478-82.

Chapter 1:

The Role of N-Linked Glycosylation in Protein Folding, Membrane Targeting, and Substrate Binding of Human Organic Anion Transporter 4 (hOAT4)

This chapter was written in the style of and published in Molecular Pharmacology (**Zhou F, Xu W, Hong M, Pan Z, Sinko PJ, Ma J, You G. Mol Pharmacol. 2005 Mar;67(3):868-76.)**

Abstract

We used a novel approach to evaluate how the addition/acquisition and processing/modification of N-linked oligosaccharides play a role in the functional maturation of human organic anion transporter hOAT4. Inhibition of acquisition of oligosaccharides in hOAT4 by mutating asparagine to glutamine and by tunicamycin treatment was combined with the expression of wild-type hOAT4 in a series of mutant Chinese hamster ovary (CHO)-Lec cells defective in the different steps of glycosylation processing. We showed that both the disruption of the glycosylation sites by mutagenesis and the inhibition of glycosylation by tunicamycin treatment resulted in a nonglycosylated hOAT4, which was unable to target to the cell surface. In contrast, hOAT4 synthesized in mutant CHO-Lec cells, carrying different structural forms of sugar moieties (mannose-rich in Lec1 cells, sialic acid-deficient in Lec2 cells, and sialic acid/galactose-deficient in Lec8 cells) were able to traffic to the cell surface. However, hOAT4 expressed in CHO-Lec1 cells had significantly lower binding affinity for its substrates compared with that expressed in parental CHO cells. This study provided novel information that addition/acquisition of oligosaccharides but not the processing of the added oligosaccharides participates in the membrane insertion of hOAT4. Processing of added oligosaccharides from mannose-rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates. Glycosylation could therefore serve as a means to specifically regulate hOAT4 function in vivo.

Introduction

Organic anion transporters (OATs) play essential roles in the body disposition of clinically important anionic drugs, including anti-human immunodeficiency virus therapeutics, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories (You, 2002). Several OAT isoforms have been cloned by us and others (You, 2004). OAT1 and OAT3 are expressed predominantly in the kidney and the brain. In the kidney, these transporters use a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit/elimination across the apical membrane into the urine. Through this tertiary transport mechanism, Na^+/K^+ ATPase maintains an inwardly directed (blood-to-cell) Na^+ gradient. The Na^+ gradient then drives a Na^+-dicarboxylate cotransporter, sustaining an outwardly directed dicarboxylate gradient that is used by a dicarboxylate/organic anion (OA) exchanger to move the OA substrate into the cell. This cascade of events indirectly links OA transport to metabolic energy and the Na^+ gradient, allowing entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell. OAT4 is present mainly in the placenta and the kidney. In the kidney, OAT4 functions as an organic anion/dicarboxylate exchanger at the apical membrane of the proximal tubule and is responsible for the reabsorption of organic anions driven by an outwardly directed dicarboxylate gradient (Ekaratanawong et al., 2004). OAT2 is predominantly expressed in the liver (You, 2002). The transport mechanism for OAT2 remains to be elucidated.

Computer modeling derived from hydropathy analysis predicted that these proteins have 12 putative membrane-spanning domains and multiple consensus sites for glycosylation and phosphorylation (You, 2002, 2004). In a previous study on the role of glycosylation in OAT1 function (Tanaka et al., 2004), our laboratory investigated the effect of disrupting the putative glycosylation sites in a mouse organic anion transporter (mOAT1) as well as its human counterpart hOAT1 by mutating asparagine to glutamine and assessing the mutant transporters in HeLa cells. One of the findings from that study is that simultaneous replacement of all asparagines in both mOAT1 and hOAT1 resulted in the transporters trapped in the intracellular compartment, suggesting that glycosylation is essential for the targeting of the transporters to the plasma membrane. The possibility cannot be excluded, however, that the amino acid substitutions introduced in that study, per se, affected the membrane insertion of the transporters.

The processing of N-linked glycosylation of proteins occurs in several steps (Fig. 1) (Kornfeld and Kornfeld, 1985). First, a dolichol pyrophosphate precursor (Glc3Man9GlcNAc2) is added to asparagine side chain of asparagine-X-serine/threonine consensus sequence for N-linked oligosaccharides in a nascent polypeptide in the endoplasmic reticulum (acquisition of N-linked oligosaccharides). Processing then begins by the removal of the three terminal glucose residues and at least one mannose residue in the endoplasmic reticulum. The partially processed polypeptide is then transported to Golgi apparatus, in which mannose residues are further trimmed and *N*-acetylglucosamine, galactose,

and sialic acid residues are sequentially added. The newly synthesized glycoproteins then exit the Golgi and are transported to their final destination.

In contrast to our previous study (Tanaka et al., 2004), which focused on the addition/acquisition step of the glycosylation through mutagenesis of N-linked glycosylation sites, the present study focused on the processing/modification steps of the glycosylation by expression of hOAT4 in a series of mutant CHO-Lec cells defect in different steps of glycosylation processing (Stanley and Siminovitch, 1977). CHO-Lec1 cells have no detectable *N*-acetylglucosaminyl-transferase I activity, and proteins expressed carry oligosaccharides bearing mannose-rich intermediates ($Man_5GlcNAc_2$) at sites normally occupied by complex carbohydrates ($Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$) in parental cell line CHO cells (Fig. 1). CHO-Lec2 and CHO-Lec8 cells lack CMP-sialic acid and UDP-galactose translocases, respectively, and are incapable of transporting CMP-sialic acid and UDP-galactose from the cytosol to the Golgi, thereby producing sialic acid-deficient ($Gal_2GlcNAc_2Man_3GlcNAc_2$, in CHO-Lec2 cells) and sialic acid-/galactose-deficient ($GlcNAc_2Man_3GlcNAc_2$, in CHO-Lec8 cells) complex oligosaccharides (Fig. 1).

Materials and Methods

[^{3}H]Estrone sulfate was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL). Parental CHO-pro5 cell line and its mutant cell lines CHO-Lec1, CHO-Lec2, and CHO-Lec8 were obtained from the American Type Culture Collection (Manassas, VA). The mutant CHO cells were originally established by Dr. Pamela Stanley (Stanley and Siminovitch, 1977). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Tunicamycin Treatment

The cells were treated with 10 μg/ml tunicamycin for 18 h. Tunicamycin inhibits transfer of dolichol pyrophosphate precursor to asparagine in the consensus sequence for N-linked glycosylation (asparagine-X-serine/threonine).

Site-Directed Mutagenesis

Mutant transporters were generated by site-directed mutagenesis of asparagine to glutamine in hOAT4. The mutant sequences were confirmed by the dideoxy chain-termination method.

Generation of Cells Stably Expressing hOAT4

Parental CHO-pro5 cells and its mutant cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8) were grown in minimal essential medium α supplemented with 10% fetal calf serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO_2 atmosphere at 37°C. Cells were seeded at 3 x 10^6/100-mm dish 24 h before transfection. For transfection of hOAT4 cDNA plasmid, a Lipofectamine 2000 reagent was used following the manufacturer's instruction. After 7 to 8 days

of selection in medium containing 2 mg/ml geneticin (G418; Invitrogen, Carlsbad, CA), resistant colonies were replated to 96 wells for cloning, expansion, and analyzing positive clones.

Transport Measurement

For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca^{2+}/Mg^{2+} (PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.3) and [^{3}H]estrone sulfate. At the times indicated in the figure legends, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquoted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values were mean ± S.E. (*n* = 3).

Isolation of Plasma Membrane Proteins by Cell-Surface Biotinylation

Cell-surface expression level of hOAT4 was examined using the membrane-impermeant biotinylation reagent NHS-SS-biotin. hOAT4 was expressed in Cos-7 cells or CHO cells in six-well plates. To initiate biotinylation, the medium was removed, and the cells were washed twice with 3 ml of ice-cold PBS/CM, pH 8.0. The plates were kept on ice, and all solutions were ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100

mM glycine and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 200 mg/ml protease inhibitor phenylmethylsulfonyl fluoride, and 3 mg/ml leupeptin, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4°C. Streptavidin-agarose beads (50 µl) were then added to the supernatant to isolate the cell-membrane protein. hOAT4 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-hOAT4 antibody.

Deglycosylation of hOAT4 with Peptide *N*-Glycosidase F and Peptide *N*-Glycosidase H

For deglycosylation, proteins from total cell lysate were denatured in 1.5% SDS and 2.5% β-mercaptoethanol, heated at 50°C for 30 min, and then incubated in 50 mM sodium phosphate buffer, pH 7.5, 1% Nonidet P-40, and 1 µl of peptide *N*-glycosidase F (PNGase F, 500 units/µl; New England Biolabs, Beverly, MA) at 37°C for 1 h. The samples were then used for immunoblotting with anti-hOAT4 antibody.

Electrophoresis and Western Blot

Protein samples (with equal amount) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS/0.05% Tween, washed, and incubated for 2 h at room temperature with polyclonal anti-hOAT4 antibody (1:500). The membranes were washed and then incubated with goat anti-rabbit IgG

conjugated to horseradish peroxidase (1:5000), and signals were detected by SuperSignal West Dura Extended Duration Substrate Kit (Pierce Chemical).

Immunofluorescence of Transfected Cells

Cells expressing hOAT4 were washed three times in phosphate-buffered saline, fixed for 20 min at room temperature in 4% Para formaldehyde in PBS, and rewashed in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. The cells were incubated for 30 min at room temperature in PBS containing 5% goat serum and then incubated for 1 h in the same medium containing anti-hOAT4 antibody (1:500) at room temperature. The cells were washed, and bound primary antibodies were detected by reaction with fluorescein isothiocynate-coupled goat anti-rabbit IgG (Chemicon International, Temecula, CA) diluted to 1:200 for 1 h. Treated cells were thoroughly washed, and the cover glasses were mounted in GEL/MOUNT (Biomeda, Foster City, CA). Samples were visualized with a Zeiss LSM-510 laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY) or a regular fluorescence microscope.

Results

Analysis of the Role of Acquisition of Oligosaccharides in hOAT4 by Site-Directed Mutagenesis.

Addition/acquisition of oligosaccharides is the first step in the glycosylation process. To determine its role in hOAT4 function, we disrupted the potential glycosylation sites by replacing asparagine with glutamine singly or in combination. We observed in Cos-7 cells that single replacement of asparagine with glutamine had no effect on cell-surface expression and transport function of hOAT4 (data not shown), suggesting that glycosylation at individual sites is not required for the transport function of hOAT4. When all of the glycosylation sites were simultaneously disrupted, the quadruple mutant protein (N39/N56/N63Q/N99Q) had a molecular mass of 47 kDa with or without the treatment of PNGase F (Fig. 2a), which removes sugar moieties from glycoproteins. This molecular mass is the same as that of nonglycosylated hOAT4, indicating that there are no additional sites for *N*-glycosylation. It is important to note that when all of the glycosylation sites were removed, the expression level of the nonglycosylated hOAT4 was almost diminished compared with that of wild-type hOAT4, although the total cellular protein of the quadruple mutant was similar to that of wild-type hOAT4 (Fig. 2, b and c). Immunofluorescence experiment (Fig. 2d) showed that the most of the quadruple mutant resided in the intracellular compartment. This result suggests that glycosylation is critical for the proper trafficking of the transporter onto the plasma membrane. Functional study (Fig. 2e) showed that there was virtually no

transport activity in the quadruple mutant-transfected cells compared with that of wild-type hOAT4-transfected cells, consistent with their cellular distributions.

To determine whether the above observations were specific to Cos-7 cells, similar studies were performed in CHO cells. Again, despite the comparable total cell expression between wild-type hOAT4 and its quadruple mutant (Fig. 3, b and c), the cell-surface expression of the quadruple mutant was almost undetectable (Fig. 3a and 3d). Therefore, the inability of the unglycosylated hOAT4 to target to the plasma membrane is an important feature of the transporter regardless of the cell type used for the studies. The quadruple mutant-transfected cells were unable to transport [^{3}H] estrone sulfate (Fig. 3e), consistent with its cellular distribution in CHO cells.

Analysis of the Role of Acquisition of Oligosaccharides in hOAT4 by the Treatment of hOAT4-Expressing Cells with Tunicamycin.

Because the possibility exists that the loss of the surface expression of nonglycosylated hOAT4 generated by replacing asparagine with glutamine (Figs. 2 and 3) may have resulted from amino acid substitutions per se rather than from deglycosylation of hOAT4, an additional experiment was performed by treating hOAT4-expressing cells with tunicamycin. Tunicamycin inhibits the first step of N-linked glycosylation (acquisition of N-linked oligosaccharides) without introducing amino acid substitution. Western blot analysis of plasma membrane proteins (Fig. 4a) showed that treatment of hOAT4-expressing cells with tunicamycin resulted in an almost complete loss of cell-surface expression of nonglycosylated hOAT4, despite that the total cell expression of nonglycosylated

hOAT4 was similar to that of fully glycosylated hOAT4 (Fig. 4b). These results confirm that acquisition of oligosaccharides indeed plays an important role in the targeting of hOAT4 to the plasma membrane. Transport activity of hOAT4 in tunicamycin-treated cells was almost undetectable (Fig. 4c).

Western Blot Analysis of the Role of Processing of Oligosaccharides in hOAT4 Expressed in Mutant CHO-Lec Cells.

Having demonstrated the role of acquisition of oligosaccharides in membrane insertion of hOAT4, we then switched our focus to the role of processing of oligosaccharides in hOAT4 function. Using clonal cell lines stably expressing the highest levels of hOAT4, we determined the molecular masses and the glycosylation patterns of hOAT4 expressed in parental CHO cells and the mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). In total cell lysates (Fig. 5), hOAT4 expressed in parental CHO cells had a molecular mass of 80 kDa. This protein was resistant to the treatment of endoglycosidase H (endo H) but sensitive to the treatment of endoglycosidase F (endo F). Endo H cleaves high mannose-containing immature N-linked carbohydrates from glycoproteins, whereas endo F cleaves both the high mannose-containing immature N-liked carbohydrates and complex-type oligosaccharides from glycoproteins. Treatment of the 80-kDa protein with endo F resulted in a reduction in its molecular size to that of nonglycosylated hOAT4. Therefore, hOAT4 expressed in parental CHO cells contained fully processed carbohydrates. hOAT4 expressed in CHO-Lec1 cells had a molecular mass of 50 kDa. This protein was sensitive to the treatment of both endo H and endo F. Treatment of the 50-kDa protein with both enzymes

resulted in a reduction in its molecular size to that of nonglycosylated hOAT4. Therefore, hOAT4 expressed in CHO-Lec1 cells was a precursor form of hOAT4 in the endoplasmic reticulum. hOAT4 expressed in CHO-Lec2 and CHO-Lec8 cells had molecular sizes of 70 or 60 kDa, respectively. These proteins were resistant to the treatment of endo H but sensitive to the treatment of endo F, and therefore probably represented sialic acid-deficient (in CHO-Lec2 cells) and sialic acid/galactose-deficient (in CHO-Lec8 cells) complex-type glycoproteins. Biotinylation of cell-surface proteins with a membrane-impermeable reagent NHS-SS-biotin (Fig. 6) showed that significant amount of hOAT4 expressed at the cell surface in both parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8). Immunofluorescence study (Fig. 7) confirmed this observation. The amount of cell-surface expression of hOAT4 (Fig. 6) correlated with its total cell expression in these cells (Fig. 5).

Functional Analysis of the Role of Processing of Oligosaccharides in hOAT4 Expressed in Mutant CHO Cells.

Transport of [^{3}H] estrone sulfate was then measured in hOAT4-expressing parental CHO cells and mutant CHO cells (Fig. 8). hOAT4 expressed in parental CHO cells, CHO-Lec2, and CHO-Lec8 exhibited transport activities that correlated with their surface expression (Fig. 6), whereas hOAT4 expressed in CHO-Lec1 exhibited much lower transport activity.

The functional properties of hOAT4 in theses cells were further characterized in several aspects. hOAT4 is known to function as an exchanger (3), with one organic anion being transported into the cells in exchange for another organic

anion being effluxed out of the cells. To determine whether modification of oligosaccharides in hOAT4 expressed in mutant CHO cells affects this functional characteristic, hOAT4-expressing parental CHO cells and mutant CHO cells were preloaded with [^{3}H]estrone sulfate followed by exposing to medium with or without dehydroepiandrosterone 3-sulfate (DHEA), another substrate for hOAT4. As shown in Table 1, significant efflux of intracellular [^{3}H] estrone sulfate was observed with hOAT4 expressed in all types of cells when cells were exposed to medium containing an exchangeable substrate DHEA, whereas little efflux was observed with the medium lacking DHEA. hOAT4 expressed in mutant CHO cells also had substrate spectra similar to that in parental CHO cells (Fig. 9). These results suggest that processing/modification of oligosaccharides in mutant CHO cells had no significant effects on the basic property of hOAT4 as an organic anion exchanger and on its substrate spectra. Finally, kinetic analysis of estrone sulfate transport in hOAT4-expressing parental CHO cells and mutant CHO cells was performed. Table 2 showed an Eadie-Hofstee analysis of the derived data. hOAT4 expressed in CHO-Lec1 cells had much lower binding affinity (K_m = 16 ± 0.3 μM) compared with hOAT4 expressed in parental CHO cells (K_m = 6.0 ± 0.5 μM). The low binding affinity of hOAT4 in CHO-Lec1 cells may contribute to the low transport activity observed in these cells (Fig. 8).

Discussion

OATs play essential roles in the body disposition of clinically important anionic drugs, including antiviral drugs, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories (You, 2002, 2004). hOAT4 and OAT1 have several distinct properties. OAT1 is localized in the basolateral membrane of the kidney proximal tubule cells and preferably transport small organic anions such as *para*-aminohippuric acid, whereas hOAT4 is localized in the apical membrane of the kidney proximal tubule cells and preferably transport bulkier organic anions such as estrone sulfate (You, 2002, 2004). One common structure feature shared among all the cloned OATs is the presence of consensus sites for N-linked glycosylation in the first extracellular loop within the current secondary structure model (You, 2002, 2004). We showed previously (Tanaka et al., 2004) that simultaneous replacement of all asparagines in OAT1 by mutagenesis impaired the trafficking of the transporter to the cell surface, suggesting an important role of glycosylation in the targeting of OAT1 onto the plasma membrane. In the present study, we showed that disruption of all the glycosylation sites in hOAT4 also rendered hOAT4 unable to traffic to the cell surface (Figs. 2 and 3). Therefore, the role of glycosylation in membrane insertion of the transporters could be a common characteristic for all members of the OAT family. However, the results obtained from the above-mentioned experiments cannot rule out the possibility that the loss of the surface expression of nonglycosylated OATs (OAT1 in the previous study and hOAT4 in the present study) generated by mutagenesis may result from amino acid substitutions per se rather than from deglycosylation of transporters. To

address this issue, we performed a new experiment in the present study by treating hOAT4-expressing cells with tunicamycin. Tunicamycin inhibits the first step of N-linked glycosylation (acquisition of N-linked oligosaccharides) without introducing amino acid substitution. We showed that the nonglycosylated hOAT4 in tunicamycin-treated cells was unable to traffic to the cell surface, confirming that acquisition of oligosaccharides indeed play a critical role in the targeting of the transporters to the cell surface. Several roles of the addition of N-linked oligosaccharides to nascent proteins have been demonstrated; for example, N-linked oligosaccharides attach to lectin-like molecular chaperons such as calnexin and calreticulin, facilitating correct protein folding, and also play a role in the "quality control" system of the endoplasmic reticulum that ensures selective transportation of the properly folded proteins for the Golgi complex (Helenius, 1994). Therefore, unfolded proteins such as nonglycosylated hOAT4 produced by site-directed mutagenesis or by tunicamycin treatment may have been trapped in the endoplasmic reticulum and was unable to target to the cell surface. The role of glycosylation in membrane insertion has been observed for proteins such as ATP-sensitive potassium channel (Conti et al., 2002), glycine transporter GLYT1 (Olivares et al., 1995), and gastric H,K-ATPase (Vagin et al., 2004). However, glycosylation is not important for proteins such as serotonin transporter (Tate and Blakely, 1994), and the sodium-dependent, purine-selective nucleoside transporter SPNT (Mangravite and Giacomini, 2003).

In contrast to our previous study (Tanaka et al., 2004) that focused on the acquisition step of the glycosylation in hOAT1 using a mutagenesis approach, the

present study focused on the processing steps of the glycosylation in hOAT4 by expressing the transporter in a series of mutant CHO-Lec cells deficient in different steps of glycosylation processing. Lec1 cells lack *N*-acetylgalactosaminyl-transferase I and hence give rise to oligosaccharides bearing high mannose intermediates. CHO-Lec2 and CHO-Lec8 cells lack CMP-sialic acid and UDP-galactose translocases and are incapable of transporting CMP-sialic acid and UDP-galactose from the cytosol to the Golgi, thereby producing sialic acid-deficient (in CHO-Lec2 cells) and sialic acid-/galactose-deficient (in CHO-Lec8 cells) complex oligosaccharides, respectively (Fig. 1). To our knowledge, such a new approach has not been used for investigating the processing/modification of oligosaccharides in any of the drug transporters cloned so far.

It is interesting that our biotinylation experiments (Fig. 6), in conjunction with immunofluorescence studies (Fig. 7), showed that hOAT4, whether containing high mannose oligosaccharides (in CHO-Lec1 cells), sialic acid-deficient oligosaccharides (in CHO-Lec2 cells), or sialic acid-/galactose-deficient oligosaccharides (in CHO-Lec8 cells), was efficiently expressed at the cell surface, suggesting that once the acquisition of oligosaccharides is accomplished, the conformational maturation of the transporter is achieved, which leads to the targeting of the transporter onto the plasma membrane. The targeting of the transporter to the cell surface may not depend on the processing of the added oligosaccharides, whereas nonglycosylated hOAT4 generated by site-directed

mutagenesis and by tunicamycin treatment may not be folded correctly and was unable to traffic to the cell surface.

We also showed that hOAT4 terminating with different types of N-linked oligosaccharides (high mannose type, sialic acid-deficient type, or sialic acid-/galactose-deficient type) preserved the basic functional properties of wild-type hOAT4 as an organic anion exchanger (Table 1). hOAT4 terminating with different types of N-linked oligosaccharides also had substrate spectra similar to those of wild-type hOAT4 (Fig. 9). Therefore, processing of the added oligosaccharides may not be critical in determining these functional properties.

However, the processing of the added oligosaccharides did affect the binding affinity of hOAT4 for its substrates. Our kinetic analysis (Table 2) revealed that hOAT4 terminating with high mannose oligosaccharides (in CHO-Lec1 cells) had significantly lower binding affinity for its substrates compared with that of hOAT4 expressed in parental CHO cells, suggesting that processing of added oligosaccharides from high mannose type to complex type strongly enhanced the ability of hOAT4 to bind its substrates, although the binding affinity may not be necessarily dependent on the completion of complex oligosaccharides, because hOAT4 with sialic acid-deficient oligosaccharides (in CHO-Lec2 cells) and sialic acid-/galactose-deficient oligosaccharides (in CHO-Lec8 cells) has binding affinities similar to those of hOAT4 expressed in parental CHO cells. On the molecular level, the presence of sialic acid and galactose at the terminus of the oligosaccharides may not contribute to the binding affinity of hOAT4 for its substrates. Rather, the *N*-acetylglucosamine, which is present in the

oligosaccharides synthesized from the parental CHO cells and from mutant cells Lec2 and Lec8 but is missing in the oligosaccharides synthesized from Lec1 cells, may play a role in the binding affinity of hOAT4 for its substrates. The 2-acetamide group in *N*-acetylglucosamine has a different orientation compared with the 2-hydroxyl group in mannose (from Lec1 cells) despite that the rest of the structures are quite similar between *N*-acetylglucosamine and mannose. Therefore, it is possible that the structure of *N*-acetylglucosamine favors the binding of hOAT4 substrates over that of mannose. Our data on hOAT4 may not be generalized to other proteins because the signaling activity for intercellular adhesion molecule 1 (Otto et al., 2004) was reduced in all of the CHO-Lec cells, and the cell-surface expression levels of thyrotrophin receptor TSHR were decreased in both Lec1 and Lec2 cells (Nagayama et al., 1998).

In conclusion, the present study provided novel information, which was not addressed in our previous study (Tanaka et al., 2004), that the addition/acquisition of oligosaccharides but not the processing of the added oligosaccharides plays a critical role in the targeting of hOAT4 to the plasma membrane. Processing of added oligosaccharides may not be essential in determining the substrate spectra of hOAT4 and its property as an organic anion exchanger. However, the processing of added oligosaccharides form mannose-rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates. Glycosylation may be altered depending on the cell type and physiological states and therefore could serve as a means to specifically regulate hOAT4 function in vivo.

Acknowledgements

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ABBREVIATIONS:

OAT, organic anion transporter; CHO, Chinese hamster ovary; OA, organic anion; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; endo, endoglycosidase; DHEA, dehydroepiandrosterone 3-sulfate; NHS-SS-biotin, biotin disulfide *N*-hydroxysuccinimide ester; h, human; m, mouse.

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Mutants	Intracellular Remaining [^{3}H]Estrone Sulfate % of Control
CHO-Wt	52.4 ± 4.0
CHO-Lec1	57.0 ± 6.4
CHO-Lec2	53.1 ± 7.4
CHO-Lec8	59.0 ± 4.0

TABLE 1 Efflux of [^{3}H] estrone sulfate mediated by hOAT4 expressed in wild-type CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8)

Cells expressing hOAT4 were preloaded with [^{3}H]estrone sulfate (500 nM) for 1 h, followed by exposure to phosphate-buffered saline (control group) or phosphate-buffered saline containing unlabeled DHEA (experimental group). The amount of intracellular [^{3}H] estrone sulfate in the experimental group was expressed as a percentage of that in the control group. The results shown are means ± S.E. (n = 3).

Cell Lines	K_m **(μ*M*)**
CHO-Wt	6.0 ± 0.5
Lec1	16.0 ± 0.3
Lec2	4.5 ± 0.6
Lec8	4.6 ± 0.45

TABLE 2 Kinetic analysis of hOAT4-mediated estrone sulfate transport in wild-type CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8)

Kinetic characteristics were determined at substrate concentrations ranging from 0.05 to 10 μM (5-min uptake) in hOAT4-expressing wild-type CHO cells and mutant CHO cells. Transport kinetic values were calculated using the Eadie-Hofstee transformation. Values given are mean ± S.E. (n = 3).

Fig 1. Scheme of N-linked oligosaccharide biosynthetic pathway in the endoplasmic reticulum and the Golgi apparatus.

The sites for disruption of glycosylation and defects in mutant CHO cells (CHO-Lec1, CHO-Lec2 and CHO-Lec8) are shown as X.

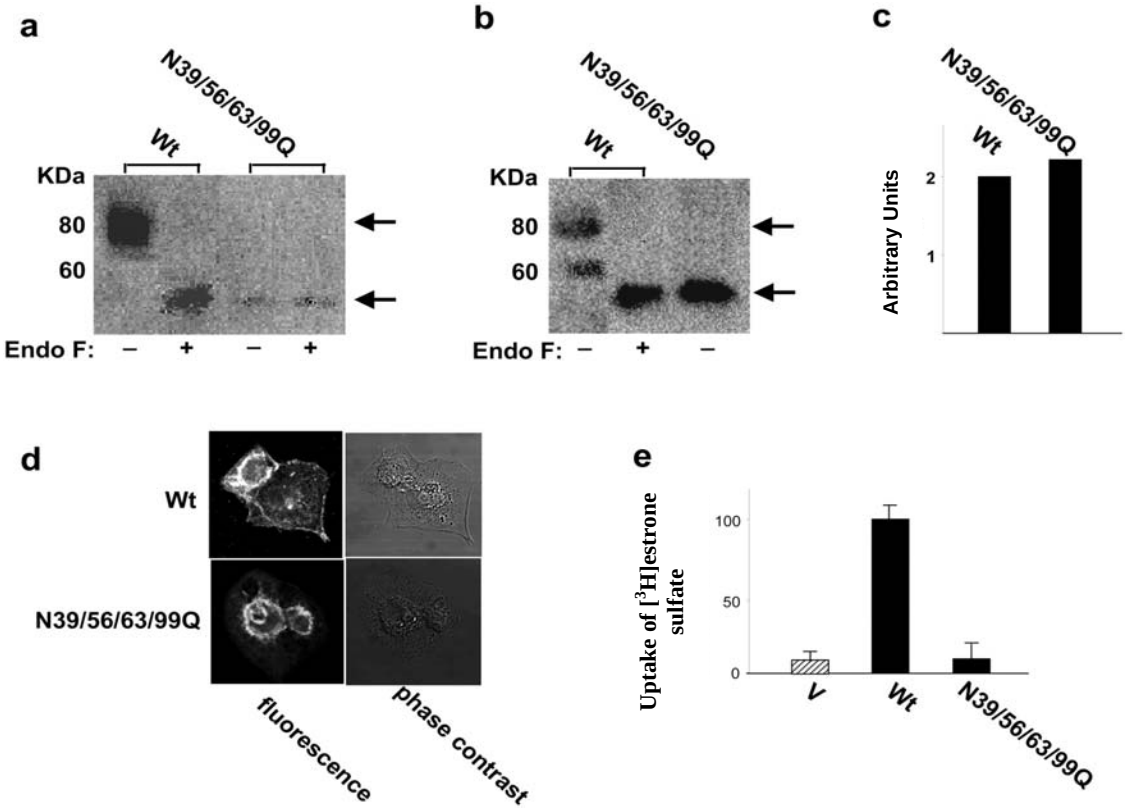

Fig 2. The effect of disrupting glycosylation sites by mutagenesis on the surface expression, cellular distribution, and the function of hOAT4 in Cos-7 cells.

a, cell-surface expression. Cos-7 cells transiently expressing wild-type (Wt) hOAT4 and its quadruple mutant (N39/56/63/99Q) were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody. **b**, total cell expression. Cells transiently expressing Wt hOAT4 and its quadruple mutant were lysed, and their proteins were separated and visualized as described above. The ~80-kDa band represented the mature form of hOAT4, and the ~60-kDa band represented the immature form of hOAT4. Treatment of the total cell lysate from Wt hOAT4-transfected cells with endo F shifted both forms to nonglycosylated form (~47 kDa).

c, quantification of the total cell expression of Wt hOAT4 and its quadruple mutant observed in Fig. 2b. The intensity of the sum for the ~80-kDa band and the ~60-kDa band in Wt hOAT4-transfected cells was similar to that of the ~47-kDa band in the quadruple mutant-transfected cells.

d, immunolocalization. Cells transiently expressing Wt hOAT4 and its quadruple mutant were stained with anti-hOAT4 antibody and fluorescein isothiocynate-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

e, [^{3}H] estrone sulfate uptake in Wt hOAT4 and its quadruple mutant-transfected cells. pcDNA vector (V)-transfected cells were used as mock control.

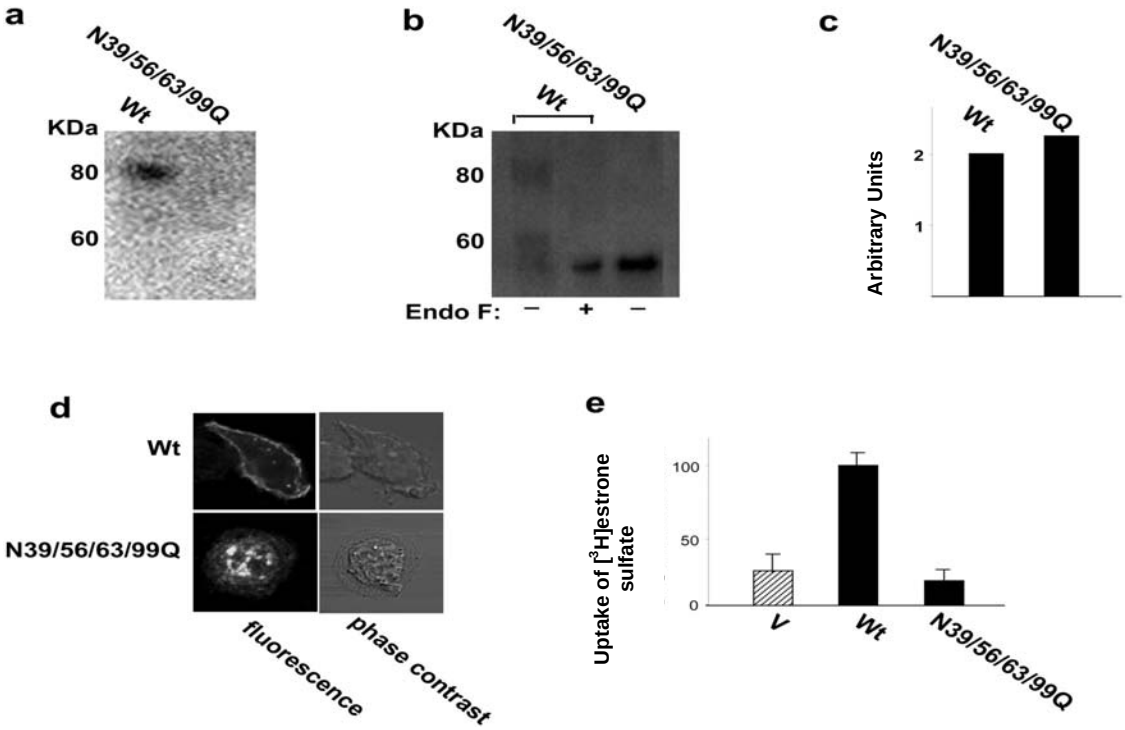

Fig 3. The effect of disrupting glycosylation sites by mutagenesis on the surface expression, cellular distribution, and the function of hOAT4 in CHO cells.

a, cell-surface expression. CHO cells transiently expressing wild-type (Wt) hOAT4 and its quadruple mutant (N39/56/63/99Q) were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody. **b**, total cell expression. Cells transiently expressing Wt hOAT4 and its quadruple mutant were lysed, and their proteins were separated and visualized as above. The ~80-kDa band represented the mature form of hOAT4, and the ~60-kDa band represented the immature form of hOAT4. Treatment of the total cell lysate from Wt hOAT4-transfected cells with endo F shifted both forms to the nonglycosylated form, ~47 kDa.

c, quantification of the total cell expression of Wt hOAT4 and its quadruple mutant observed in Fig. 3b. The intensity of the sum for the ~80-kDa band, the ~60-kDa

band, and the ~47-kDa band in Wt hOAT4-transfected cells was similar to that of ~47-kDa band in the quadruple mutant-transfected cells.

d, immunolocalization. CHO cells transiently expressing Wt hOAT4 and its quadruple mutant (N39/56/63/99Q) were stained with anti-hOAT4 antibody and fluorescein isothiocynate-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

e, [^{3}H] estrone sulfate uptake in Wt hOAT4- and its quadruple mutant-transfected cells. pcDNA vector (V)-transfected cells were used as mock control.

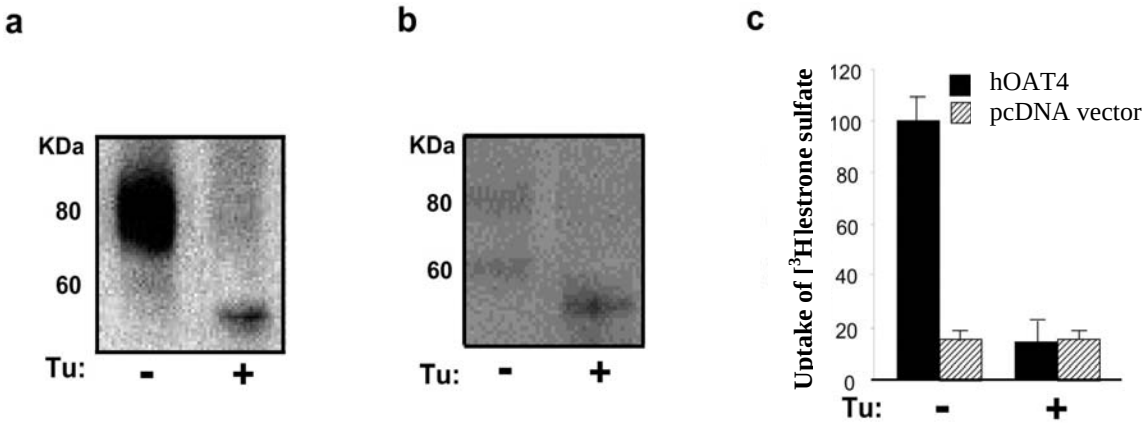

Fig 4. The effect of inhibition of glycosylation by tunicamycin treatment on the cell-surface expression and the function of hOAT4.

a, cell-surface expression. Cos-7 cells expressing wild-type (Wt) hOAT4 were treated with or without tunicamycin (Tu) followed by biotinylation. The labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody.

b, total cell expression. Cells expressing Wt hOAT4 were treated with or without tunicamycin, lysed, and their proteins were separated and visualized as above.

c, [^{3}H] estrone sulfate uptake in hOAT4-transfected cells treated with or without tunicamycin. pcDNA vector (V)-transfected cells were used as mock control.

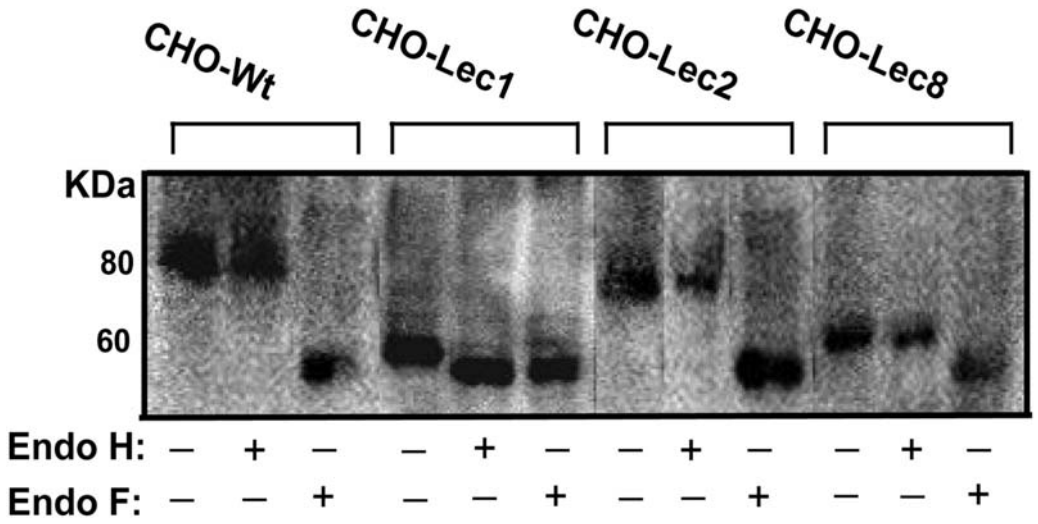

Fig 5. Western blot analysis of the expression pattern of hOAT4 in total cell lysates from parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8).

Total cell lysate proteins isolated from hOAT4-expressing parental CHO cells and mutant CHO cells were treated with endo H or endo F, followed by Western blotting with anti-hOAT4 antibody.

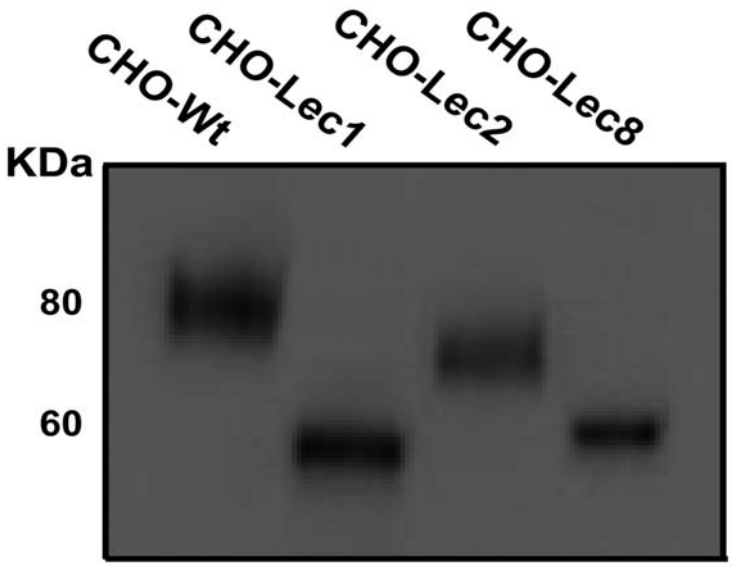

Fig 6. Western blot analysis of cell-surface expression of hOAT4 expressed in parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8).

Cells expressing hOAT4 were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads and separated by SDS-PAGE followed by Western blotting with anti-hOAT4 antibody.

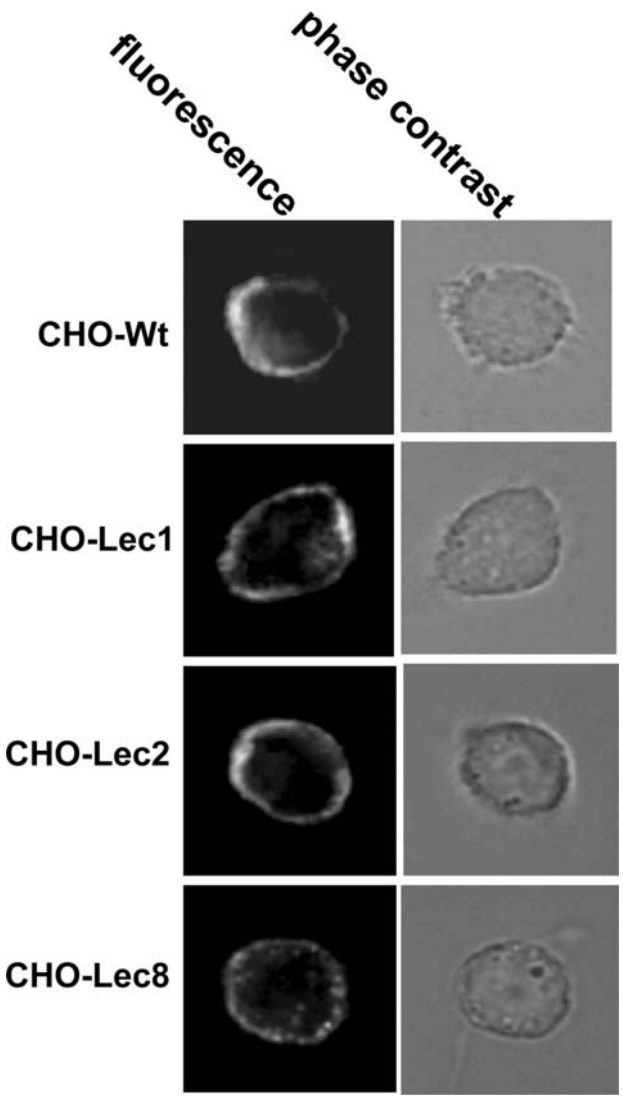

Fig 7. Immunofluorescence of hOAT4 expressed in parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8).

Cells expressing hOAT4 were stained with anti-hOAT4 antibody and fluorescein isothiocynate-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

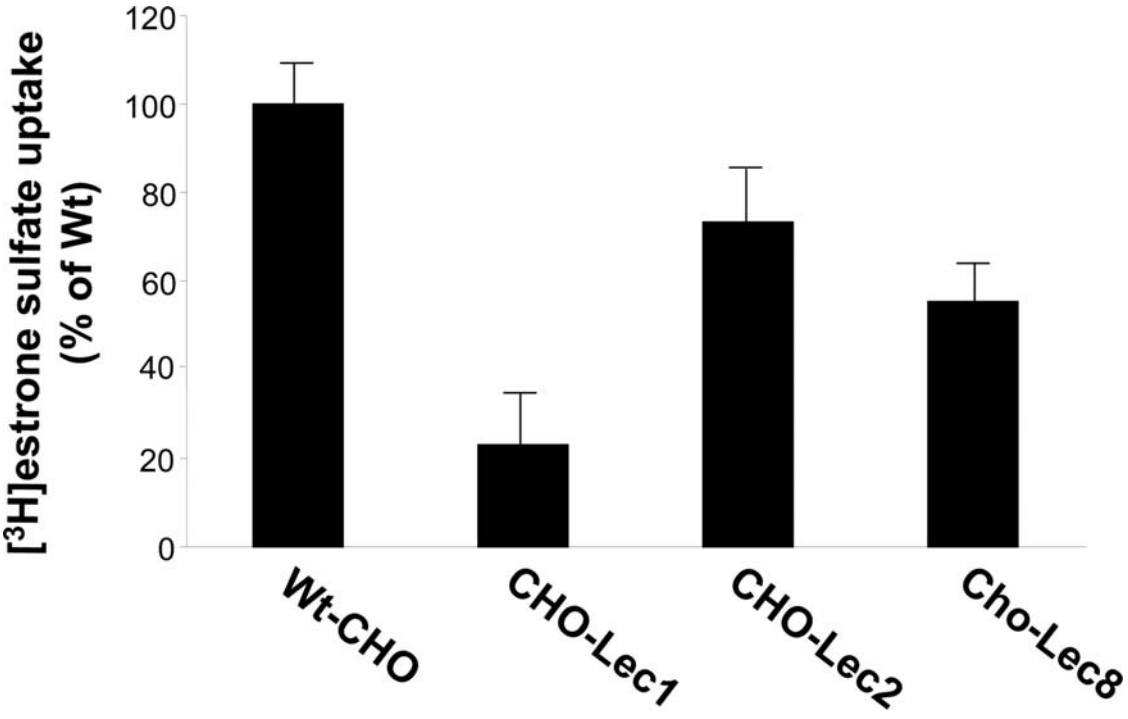

Fig 8. [^{3}H] Estrone sulfate uptake into hOAT4-expressing parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8).

[^{3}H] Estrone sulfate uptake (100 nM and 5 min) was measured in hOAT4-expressing parental and mutant CHO cells. The data are presented as the percentage of uptake in parental CHO cells. Values given are mean ± S.E. (*n* = 3).

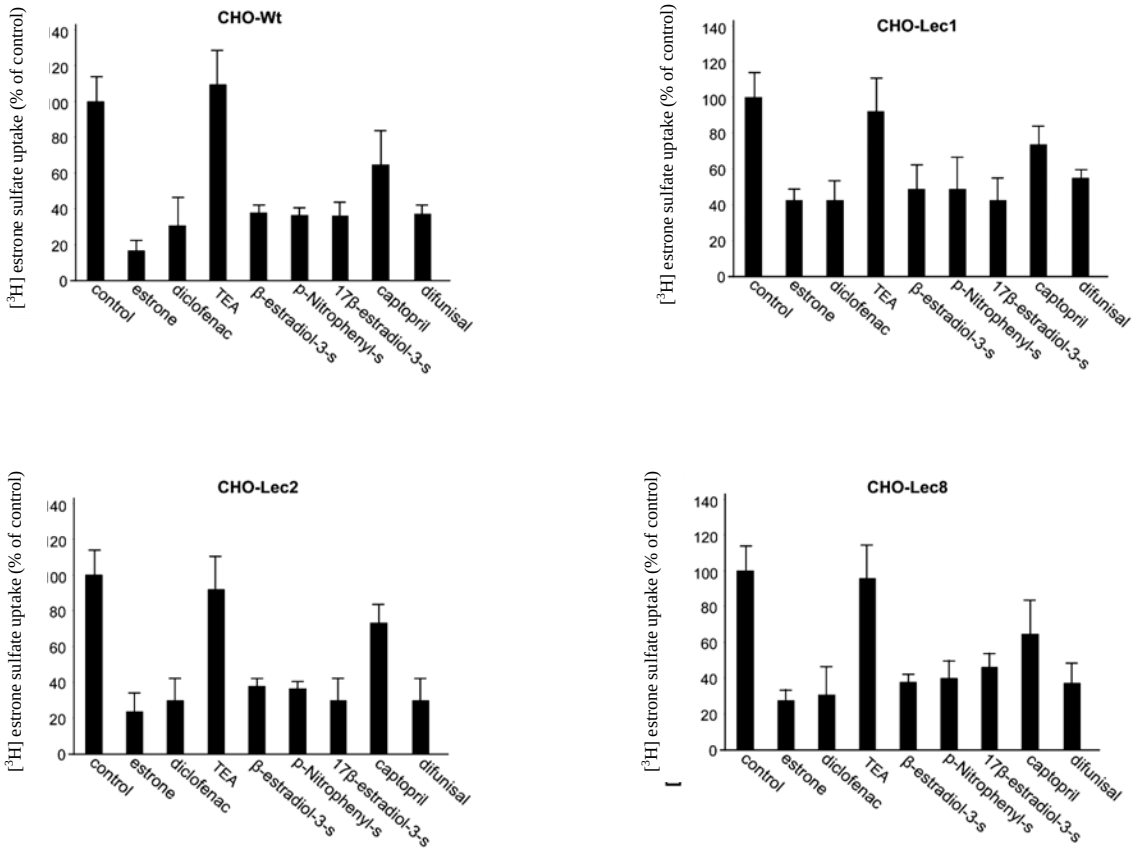

Fig 9. Substrate selectivity of hOAT4 expressed in parental CHO cells and mutant CHO cells (CHO-Lec1, CHO-Lec2, and CHO-Lec8).

The substrate selectivity was obtained in competition experiments. Uptake of [^{3}H] estrone sulfate (100 nM) was measured in the absence (control) and presence of various unlabeled compounds (1 μM). The data are presented as the percentage of control uptake. Values given are mean ± S.E. (*n* = 3).

Chapter 2:

The Role of Glycine Residues in the Function of Human Organic Anion Transporter 4

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Abstract

Human organic anion transporter 4 (hOAT4) belongs to a superfamily of organic ion transporters that play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories. In this study, we investigated the role of conserved glycine residues in hOAT4 function. We mutagenized each of the six glycine residues (at positions 11, 241, 383, 388, 400, and 466) to serine, and their functional properties were analyzed in COS-7 cells by measuring the uptake of [^{3}H] estrone sulfate. Our results showed that mutants G11S, G383S, G388S, and G466S exhibited transport activities comparable with those of wild-type hOAT4. In contrast, mutants G241S and G400S almost completely lost transport function. We then further characterized Gly-241 and Gly-400 by mutagenizing these residues to amino acids with varying sizes of side chains, including alanine, valine and leucine. We demonstrated that increasingly larger side chains at positions 241 and 400 increasingly impaired hOAT4 function. Cell-surface biotinylation using an impermeant biotinylation reagent showed that mutations of Gly-241 and Gly-400 interfered with the trafficking of the transporter onto cell surface. Immunofluorescence analysis of mutant-transfected cells confirmed these results. Substitutions of amino acids with large side chains at positions 241 and 400 resulted in decreased V_{max} and increased $K_{m.}$ These results suggest that Gly-241 and Gly-400 are important both in targeting the transporter to the plasma membrane and in substrate binding. This is the first identification and characterization of critical amino acid residues in hOAT4 and may provide

important insights into the structure-function relationships of the organic ion transporter family.

Introduction

Organic ion transporters play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, antitumor drugs, antibiotics, antihypertensives, and anti-inflammatories (Dresser et al., 2001; You, 2002; Koepsell et al., 2003). Organic anion transporters (OATs) and organic cation transporters (OCTs) are two major classes of the organic ion transporter family. So far, four members of the OAT class (OAT1, OAT2, OAT3, and OAT4) and three members of the OCT class (OCT1, OCT2, and OCT3) have been cloned from various species. Although both classes have distinct substrate preferences, with OATs for those carrying negative charges and OCTs for those carrying positive charges, they share several common features: similar predicted transmembrane topology; expression in tissues as varied as kidney, liver, intestine, brain, and placenta; and interaction with numerous structurally and pharmacologically diverse compounds. A hydrophobic backbone and a charge are common requirements of OATs and OCTs for their substrates. An examination of the amino acid sequences of organic ion transporters across species from human to *Caenorhabditis elegans* revealed six glycine residues that are evolutionarily conserved. We hypothesize that these conserved residues may play critical roles in the function of organic ion transporters. In this study, we used human organic anion transporter 4 (hOAT4) as an experimental model to test this hypothesis.

hOAT4 is an organic anion transporter recently cloned and localized to kidney and placenta (Cha et al., 2000; Ugele et al., 2003). Kidney is one of the major organs responsible for the secretion of endogenous metabolites and xenobiotics,

including drugs and environmental toxins, from the body. Placenta forms the sole structural barrier between the mother and the developing fetus. It mediates the transfer of nutrients from the mother to the fetus and eliminates metabolic waste products from the fetus. Multiple transporters in kidney and placenta participate in these processes. Functional characterization in *Xenopus laevis* oocytes and cultured cells revealed that hOAT4 transports steroid sulfates, such as estrone sulfate and dehydroepiandrosterone-3-sulfate (a major circulating steroid secreted by the adrenal cortex), as well as drugs, such as nonsteroidal anti-inflammatory drugs, antitumor drugs, and environmental toxins (Cha et al., 2000; Babu et al., 2002; Khamdang et al., 2002; Takeda et al., 2002; Ugele et al., 2003). Based on its tissue localization and function, it is therefore proposed that hOAT4 may involve the body disposition of these compounds through kidney and placenta. Despite these crucial roles, the structure-function relationships of hOAT4 have not been elucidated. Using combined approaches of site-directed mutagenesis, functional assay, cell-surface biotinylation, and immunofluorescence analysis, we provide in this study the first identification and characterization of critical amino acid residues in hOAT4 that are important for its transport function.

Materials and Methods

[^{3}H] Estrone sulfate, [^{3}H] dehydroepiandrosterone 3-sulfate (DHEAS), and [^{14}C] *para*-aminohippurate ([^{14}C] PAH) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Culture media were obtained from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Generation of hOAT4 and hOAT4-myc cDNAs

hOAT4 cDNA (Genbank accession number AB026198) was amplified by PCR from human placenta cDNA library (BD Biosciences Clontech, Palo Alto, CA). The primers for PCR were designed from the published sequences. The pair of primers was 5'-AGCTGCTAGCTCCAAACAGCAGTTAGGTCAGC-3' (sense) and 5'-AGCTAAGCTTTGACTAAAGGGGCTCCATGC-3' (antisense). The cDNA was then subcloned into pcDNA 3.1 vector. hOAT4-myc cDNA encodes a fusion protein consisting of full-length hOAT4 with 10 amino acids of the human c-Myc epitope (EQKLISEEDL) at the carboxyl terminus. The myc-tagged hOAT4 cDNA was synthesized by PCR. The sequences were confirmed by the dideoxy chain termination method.

Site-Directed Mutagenesis

Specific amino acid changes were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The sequences of the oligonucleotides used in the mutagenesis

procedure are shown in Table 1. The mutants were confirmed by the dideoxy chain termination method.

Expression in COS-7 Cells

COS-7 cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were transfected with plasmid DNA (hOAT4 wild type, hOAT4 mutants, or pcDNA vector) using LipofectAMINE 2000 reagent (Invitrogen) following the manufacturer's instructions. Twenty-four hours after transfection, the transport activities were measured.

Transport Measurements

Uptake of [^{3}H] estrone sulfate was initiated by adding uptake solution (PBS, pH 7, containing 5 mM glucose and 100 nM [^{3}H] estrone sulfate). At the times indicated in the figures, the uptake was stopped by rapidly washing the cells with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquoted for liquid scintillation counting. Uptake count was standardized by the amount of protein in each well. Values were mean ± S.E. (n = 3).

Cell-Surface Biotinylation

Cell-surface expression levels of hOAT4 and its mutants were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Pierce Chemical, Rockford, IL). The transporters were expressed in COS-7 cells in six-well plates using LipofectAMINE 2000 as described above. After 20 h, the medium was

removed, and the cells were washed twice with 3 ml of ice-cold PBS, pH 8.0. The plates were kept on ice, and all solutions were kept ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS containing 100 mM glycine, then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 µl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and protease inhibitors phenylmethylsulfonyl fluoride, 200 µg/ml, and leupeptin, 3 µg/ml, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4°C. Streptavidin-agarose beads (50 µl; Pierce Chemical) were then added to the supernatant to isolate cell membrane protein. hOAT4 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-hOAT4 antibody (Alpha Diagnostic International, Inc., San Antonio, TX).

Electrophoresis and Immunoblotting

Protein samples were loaded (40 µg/lane) on 7.5% SDS-PAGE minigels and electrophoresed using a mini cell (Bio-Rad, Hercules, CA). Proteins were transferred to PVDF membranes in an electro-elution cell (Bio-Rad). The blots were blocked for 1 h with 5% nonfat dry milk in PBS-Tween (80 mM Na_2HPO_4, 20 mM KH_2PO_4, 100 mM NaCl, and 0.05% Tween 20, pH 7.5), washed, and incubated overnight at 4°C with anti-hOAT4 antibody (2 µg/ml; Alpha Diagnostic International, Inc.). The membranes were washed, incubated with goat anti-rabbit IgG

conjugated to horseradish peroxidase (1:15000), and signals were detected by SuperSignal West Dura extended duration substrate kit (Pierce Chemical).

Immunofluorescence of Transfected Cells

Seventy-two hours after transfection, COS-7 cells were washed three times in PBS, fixed for 15 min at room temperature in 4% Para formaldehyde in PBS, and rewashed in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. After that, the cells were incubated for 15 min at room temperature in PBS containing 5% goat serum and then incubated for 1 h in the same medium containing anti-hOAT4 antibody (3μg/ml) at room temperature. The cells were washed, and bound primary antibodies were detected by reaction with fluorescein isothiocynate-coupled goat anti-rabbit IgG (Chemicon International, Temecula, CA) diluted 1:200 for 1 h. Cells were thoroughly washed, and the cover glasses were mounted in GEL/MOUNT (Biomeda, Foster City, CA). Samples were visualized with a Zeiss LSM-510 laser scanning microscope (Carl Zeiss Inc., Thornwood, NY).

Statistics

To test the significance of differences between data sets, Student's *t* test was performed.

Results

Immunoblot Analysis of hOAT4.

To our knowledge, there has been no report on the detection of hOAT4 protein by immunoblot analysis using anti-hOAT4 antibody. Therefore, the expression of hOAT4 protein in COS-7 cells was first examined by immunoblot analysis of cell-surface proteins (Fig. 1). Cell-surface proteins were biotinylated with the membrane-impermeant reagent NHS-SS-biotin, isolated with streptavidin-coated beads, and immunoblotted with anti-hOAT4 antibody. Anti-hOAT4 was raised in rabbit against a synthetic peptide corresponding to the 18 amino acids of the COOH terminus of hOAT4. The antibody recognized a product with a molecular mass of ~83 kDa in hOAT4-transfected cells (Fig. 1a, lane 1) but not in pcDNA vector-transfected cells (Fig. 1a, lane 2; mock control). To further confirm that this product was hOAT4, we engineered an epitope tag (c-myc) at the C terminus of the hOAT4 protein, allowing the detection of hOAT4 protein using anti-myc antibody. Anti-myc antibody recognized a product (Fig. 1a, lane 3) that has the same molecular mass as that detected by hOAT4 antibody. The predicted molecular mass for hOAT4 from its amino acid sequence is ~60 kDa. The higher molecular mass of 83 kDa observed in this study may represent the glycosylated form of hOAT4. To test this possibility, we treated the cell-surface proteins from hOAT4-transfected cells with PNGase F, an enzyme specifically cleaving *N*-linked oligosaccharide chains (Fig. 1b). After the treatment, a 60-kDa product was then detected on the immunoblot. These results indicate that hOAT4 is modified by abundant *N*-glycosylation in COS-7 cells. Importantly, these results demonstrate

the feasibility of using anti-hOAT4 antibody for the following structure-function studies.

Uptake of [^{3}H] Estrone Sulfate by Wild-Type hOAT4.

Because this is the first study using COS-7 cells as an expression system for hOAT4, the functional properties of hOAT4 in these cells were characterized. The time course for [^{3}H] estrone sulfate uptake was compared in vector-transfected (Fig. 2, ○) and hOAT4-transfected (Fig. 2, ●) cells. As expected, uptake was markedly faster into hOAT4-expressing cells. In hOAT4-expressing cells, uptake increased linearly for approximately 5 min and reached a steady state between 10 and 20 min. Therefore, an uptake period of 3 min (initial rate) was chosen for future studies.

To evaluate the substrate selectivity, we examined several radiolabeled compounds in terms of whether they are taken up into COS-7 cells via hOAT4. As shown in Fig. 3, [^{3}H] estrone sulfate (Fig. 3a) and DHEAS (Fig. 3b) were transported into the cells. No uptake of [^{14}C] PAH was detected (Fig. 3c). hOAT4 is known to function as a Na^+-independent transporter in other systems (Cha et al., 2000). Therefore, the role of Na^+ in hOAT4-mediated estrone sulfate uptake in COS-7 cells was investigated (Fig. 4). This was done by examining the effect of isosmotically replacing Na^+ (137 mM) in the incubation buffer with the monovalent cation choline (137 mM choline chloride). The result showed that replacement of Na^+ by choline had no effect on estrone sulfate uptake, demonstrating that the transport function is Na^+-independent. The above functional characteristics of

hOAT4 (substrate specificity and Na^+-independence) were consistent with those obtained from other systems (Cha et al., 2000), suggesting that hOAT4-transfected COS-7 cells are a valid model system for the structure-function analyses.

Uptake of [^{3}H] Estrone Sulfate by Glycine Mutants.

To explore the functional role of the evolutionarily conserved glycine residues, we mutated each glycine (at positions 11, 241, 383, 388, 400, and 466) to serine. The functional properties of these mutants were then determined by measuring the uptake of [^{3}H] estrone sulfate in mutant-transfected cells. As shown in Fig. 5, mutants G11S, G383S, G388S, and G466S exhibited significant transport activities compared with those of wild-type hOAT4. In contrast, mutants G241S and G400S almost completely lost transport function.

To further elucidate the molecular mechanisms underlying the effects of mutations at Gly-241 and Gly-400, we mutagenized these two residues to amino acids with varying sizes of side chains, including alanine, valine, and leucine. As shown in Fig. 6, substitution of Gly-241 (Fig. 6a) and Gly-400 (Fig. 6b) with amino acids with small side chains, such as alanine, resulted in detectable transport activities, although they were much reduced compared with those of wild-type hOAT4. In contrast, substitution of Gly-241 and Gly-400 with amino acids with larger side chains, such as valine and leucine, rendered hOAT4 nonfunctional. Mutants G241S and G400S were also included in this study and shown to have little transport activity. This finding clearly demonstrates that increasingly larger side chains at positions 241 and 400 increasingly impair hOAT4 function.

Immunoblot Analysis on the Expression of Glycine Mutants.

The reduced, or lack of, transport activities of Gly-241 and Gly-400 mutants could be caused by changes in the absolute number of transporters, turnover rate, substrate binding affinity, or a combination of these factors. As a first step in evaluating possible changes, we compared the protein expression levels of wild-type hOAT4 and its mutants in the total cell extracts and on the cell surface by immunoblot analysis (Fig. 7). We demonstrated above (Fig. 1) that hOAT4 in COS-7 cells is a glycosylated protein with a molecular mass of ~83 kDa. In total cell extracts (Fig. 7, a and b, bottom), all of the hOAT4 mutants expressed a band at ~83 kDa similar in intensity to that expressed by wild-type protein, suggesting that similar amounts of wild-type and mutant proteins are expressed in these cells. In contrast, the intensity of the 83-kDa band detected from the cell-surface pools decreased significantly in mutant-transfected cells compared with that of wild-type hOAT4-transfected cells (Fig. 7, a and b, top): the larger the side chains at positions 241 and 400, the less the expression of the mutants at the cell surface. Figure 7, c and d, shows the densitometry analyses of the surface expression for Fig. 7a and 7b, respectively.

Immunofluorescence Analysis on the Expression of Glycine Mutants.

Further evidence of the difficulty in transporting mutant protein to the plasma membrane was obtained by immunofluorescence (Fig. 8). Although the plasma membrane was clearly labeled (shown as green fluorescence) in cells transfected with wild-type hOAT4, fluorescence remained mainly in the intracellular

compartment in cells transfected with mutants G241S, G241V, G241L (Fig. 8a) and G400S, G400V, and G400L (Fig. 8b). G241A- and G400A-transfected cells showed both surface expression and accumulation in the intracellular compartment. Phase contrast images showed that cells were fully attached to the culture dishes under all conditions. From all these data, it can be concluded that larger side chains at positions 241 and 400 impaired the proper targeting of hOAT4 to the plasma membrane.

Kinetic Analysis of Estrone Sulfate Transport Mediated by Mutants G241A and G400A.

To further examine the mechanisms underlying the reduced transport activity of hOAT4 by substitution of Gly-241 and Gly-400 with alanine, we determined [^{3}H] estrone sulfate uptake at different substrate concentrations. An Eadie-Hofstee analysis of the derived data (Fig. 9) showed that alanine substitution of these residues resulted in decreased V_{max} (53.8 ± 1.1 pmol/μg · 2 min with wild-type hOAT4, 21.2 ± 0.5 pmol/μg · 2 min with G241A, and 25.3 ± 1.7 pmol/μg · 2 min with G400A) and increased K_m (3.2 ± 0.1 μM with wild-type hOAT4, 5.2 ± 0.4 μM with G241A, and 6.3 ± 0.7 μM with G400A).

Discussion

The vital importance of organic ion transport in body drug disposition is clear (Dresser et al., 2001; You, 2002; Koepsell et al., 2003). However, our knowledge about the structure-function relationships of organic ion transporters is minimal. The aim of this study was to identify the structural determinants important for the function of hOAT4. To this end, we explored the roles of six glycine residues (at positions 11, 241, 383, 388, 400, and 466) in hOAT4. These glycine residues are conserved across organic ion transporters of different origin, from human to *C. elegans*. This led to our hypothesis that they may play important roles in the function of these transporters.

By first mutagenizing each of these glycine residues to serine, we found that mutants G11S, G383S, G388S, and G466S transported significant amounts of estrone sulfate. In contrast, mutants G241S and G400S lost ability to transport this molecule, suggesting an important role of Gly-241 and Gly-400 in hOAT4 function. To further investigate the significance of Gly-241 and Gly-400, we then mutagenized these two residues to several other amino acids with varying side chains, such as alanine, valine, and leucine. We showed that the size of the side chain of the residues at positions 241 and 400 is of functional significance provided no charge is present. When native Gly-241 and Gly-400 were replaced with alanine, a residue with a side chain slightly larger than that of glycine and equally neutral, the resulting hOAT4 molecules (G241A and G400A) still exhibited measurable transport activities, although much reduced compared with that of wild-type hOAT4. In contrast, when native Gly-241 and Gly-400 were replaced

with valine or leucine, residues with consecutively larger side chains than alanine and devoid of charge, the resulting hOAT4 molecules (G241V, G241L, G400V, and G400L) became nonfunctional.

The reduced or lack of transport activity of glycine mutants could result from the reduced or lack of expression of the mutant transporter protein or could result from impaired binding abilities of the mutants for their substrates. By directly measuring both total cell and cell surface expression of these mutants, we showed that despite the similar total cell expression of these mutants relative to that of wild-type hOAT4, larger side chains at positions 241 and 400 resulted in a decrease in the expression of the mutants at the cell surface. These results suggest that substitution of Gly-241 and Gly-400 with larger side chains does not interfere with the total cell expression of the transporter protein but rather interferes with the trafficking of the transporter to the plasma membrane. Our immunofluorescence study confirmed these results. In addition to the much lower cell-surface expression, our kinetic analysis showed that the reduced transport activity was also contributed to by a reduced binding affinity of the mutants for its substrates. Substitution of Gly-241 and Gly-400 with alanine resulted in a 1.6- to 2-fold increase in K_m compared with that of wild-type hOAT4. Based on the predicted 12-transmembrane topology of hOAT4, Gly-241 and Gly-400 reside in transmembrane domain 5 and at the mouth of transmembrane domain 8, respectively. Glycine has been implicated to play an important role in stabilizing transmembrane helix-helix association for membrane proteins such as water channel aquaporin-1 (Murata et al., 2000; Russ and Engelman, 2000). Therefore,

our data suggest that transmembrane domains 5 and 8 in hOAT4 may form part of the translocation pathway for the substrates.

In conclusion, mutation of glycine at positions 241 and 400 impairs hOAT4 function by impairing both the trafficking of the transporter to the cell surface and its binding ability for the substrates. The extent of the impairment is dependent on the size of the side chains at positions 241 and 400. The increasingly larger side chains increasingly impair hOAT4 function. This is the first identification and characterization of critical amino acid residues in hOAT4 and may provide important insights into the structure-function relationships of the organic ion transporter family.

ABBREVIATIONS

OAT, organic anion transporter; OCT, organic cation transporter; hOAT4, human organic anion transporter 4; DHEAS, [^{3}H]dehydroepiandrosterone 3-sulfate; [^{14}C]PAH, [^{14}C]*para*-aminohippurate; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

References

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Mutation	igonucleotides (5'→3')
G11S	\G CAA GCC AGC GGC GTG GGC CTC TTC
G241S	T CAG CGC AAG CCA GGC GGC GCT GGG
G382S	:A GGC CCT CTT CAG CGC CGT GGA CTT CCT
G388S	G GAC TTC CTG AGC CGG GCC ACC ACT
G400S	C AGT TTC CTT AGC CGC CGC ACC ATC C
G466S	GG ATG ACA GCA GAT AGC ATT CTG CAT ACA GTG
G241A	T CAG CGC AGC CCA GGC GGC GCT GGG
G241L	T CAG CGC ACT CCA GGC GGC GCT GGG
G241V	T CAG CGC AGT CCA GGC GGC GCT GGG
G400A	C AGT TTC CTT GCC CGC CGC ACC ATC C
G400L	C AGT TTC CTT CTC CGC CGC ACC ATC CAG GC
G400V	C AGT TTC CTT GTC CGC CGC ACC ATC C

TABLE 1 Oligonucleotides used for mutagenesis

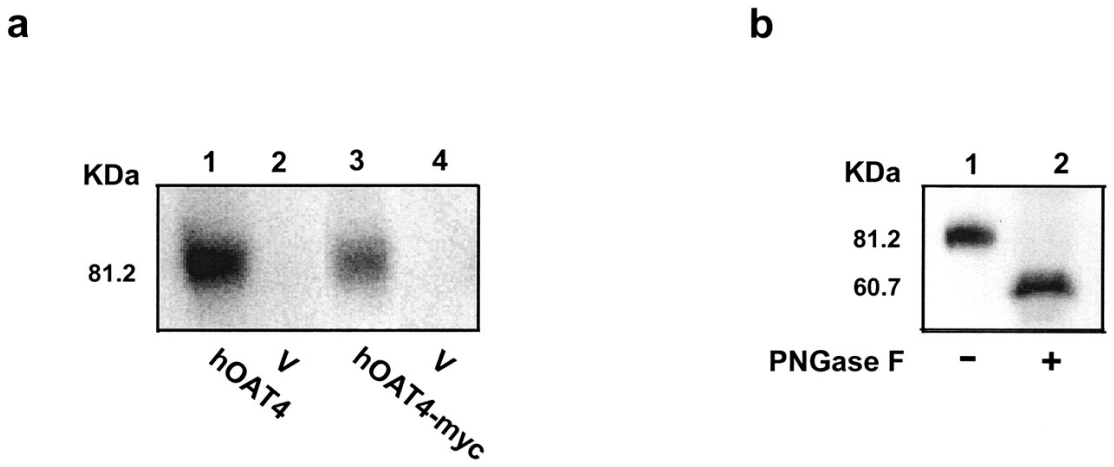

Fig 1. Immunoblot analysis of hOAT4 expressed at cell surface.

Cell-surface proteins were isolated through biotinylation from cells transfected with hOAT4 (lane 1), hOAT4-myc (lane 3), or pcDNA vector (V) (lanes 2 and 4), separated by SDS-PAGE, and transferred to PVDF membrane (a). The blots were then probed with anti-hOAT4 antibody (lanes 1 and 2) or anti-myc antibody (lanes 3 and 4), followed by horseradish peroxidase-labeled secondary antibody. Cell-surface proteins isolated through biotinylation from hOAT4-transfected cells were treated with (lane 2) or without (lane 1) PNGase F, separated by SDS-PAGE, and transferred to PVDF membrane (b). The blots were then probed with anti-hOAT4 antibody, followed by horseradish peroxidase-labeled secondary antibody.

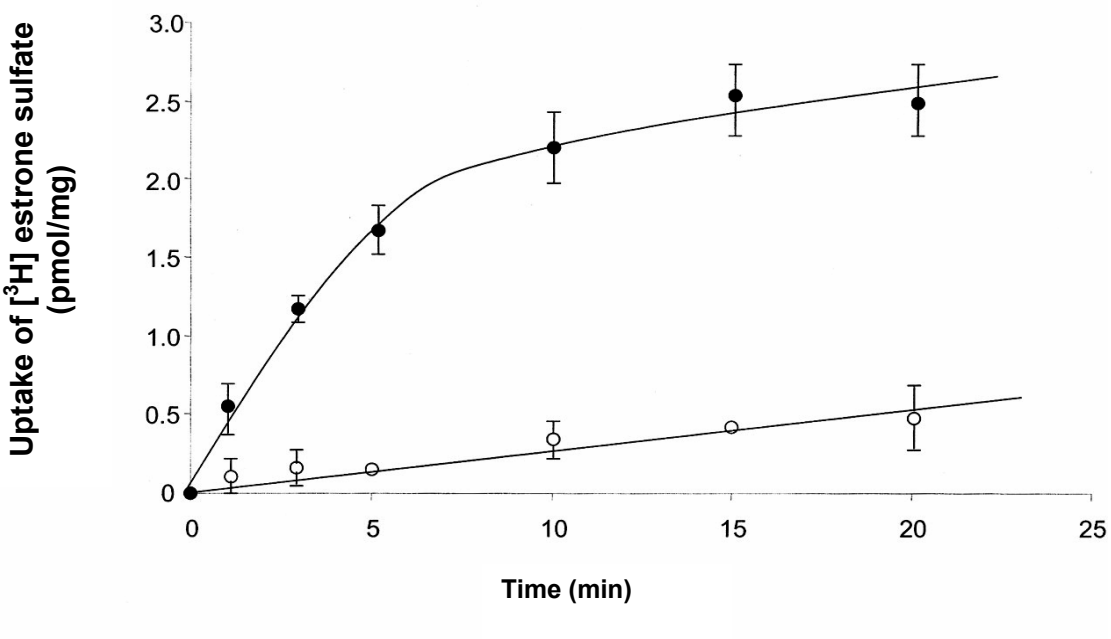

Fig 2. Time course of [^{3}H] estrone sulfate uptake in COS-7 cells.

[^{3}H] estrone sulfate (100 nM) was added to the cells transfected with hOAT4 (●) and vector (○). At specified time points, cells were washed twice with ice-cold PBS, lysed with 0.2 N NaOH, neutralized with 0.2 N HCl, and measured for radioactivity. Each value represents the mean ± S.D. of three experiments.

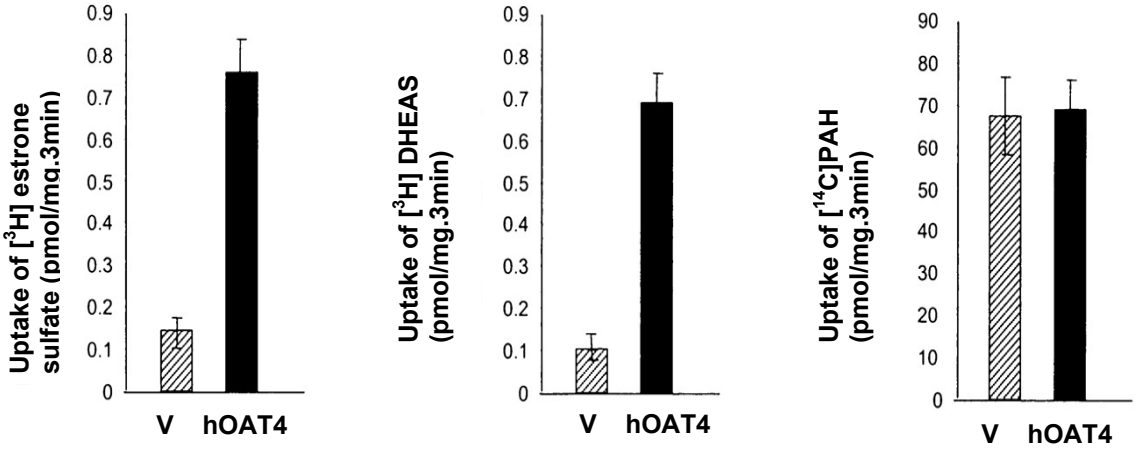

Fig 3. Substrate specificity.

The uptake of [^{3}H] estrone sulfate, [^{3}H] DHEAS, and [^{14}C] PAH were measured in COS-7 cells transfected with hOAT4 (solid bars) and vector (shaded bars). Each value represents the mean ± S.D. of three experiments.

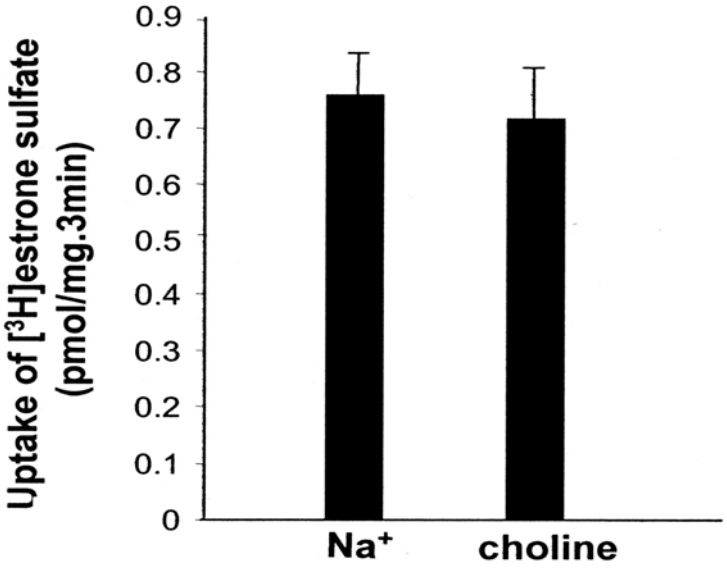

Fig 4. Effect of extracellular Na^+ on uptake of estrone sulfate.

Uptake of [^{3}H] estrone sulfate (100 nM) after a 3-min incubation was measured in the presence or absence of Na^+ (Na^+ was replaced by choline). Each value represents the mean ± S.D. of three experiments.

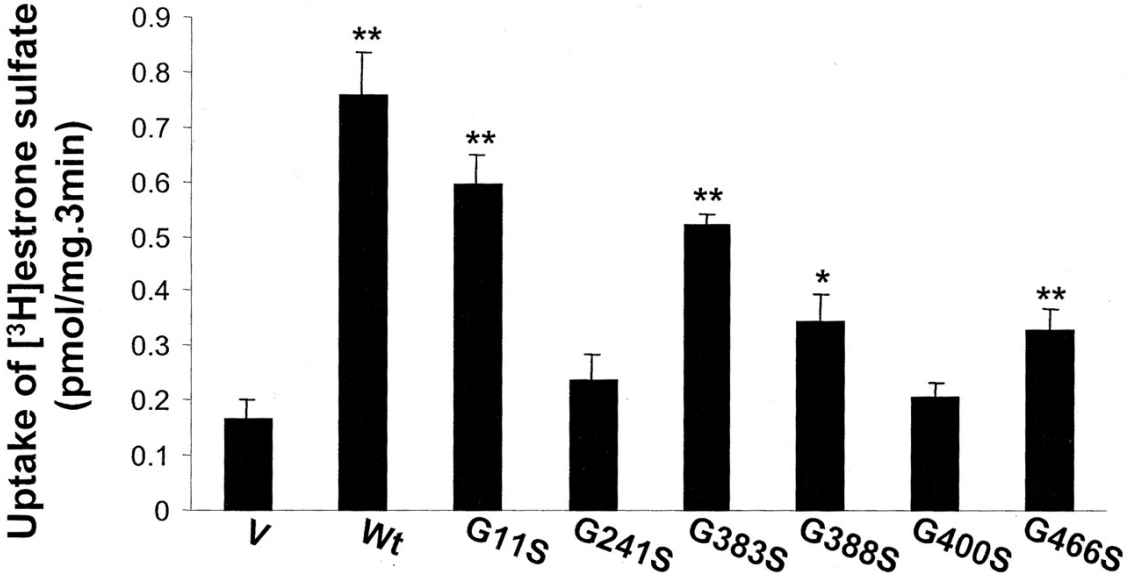

Fig 5. Uptake of [^{3}H] estrone sulfate by cells expressing glycine mutants.

Glycine (G) at positions 11, 241, 383, 388, 400, and 466 was individually mutagenized to serine (S), and the transport of 100 nM [^{3}H] estrone sulfate in mutant-transfected cells was measured. V, pcDNA vector. Values were mean ± S.E. (n = 3).*, $P < 0.05$ and **, $P < 0.01$ versus control.

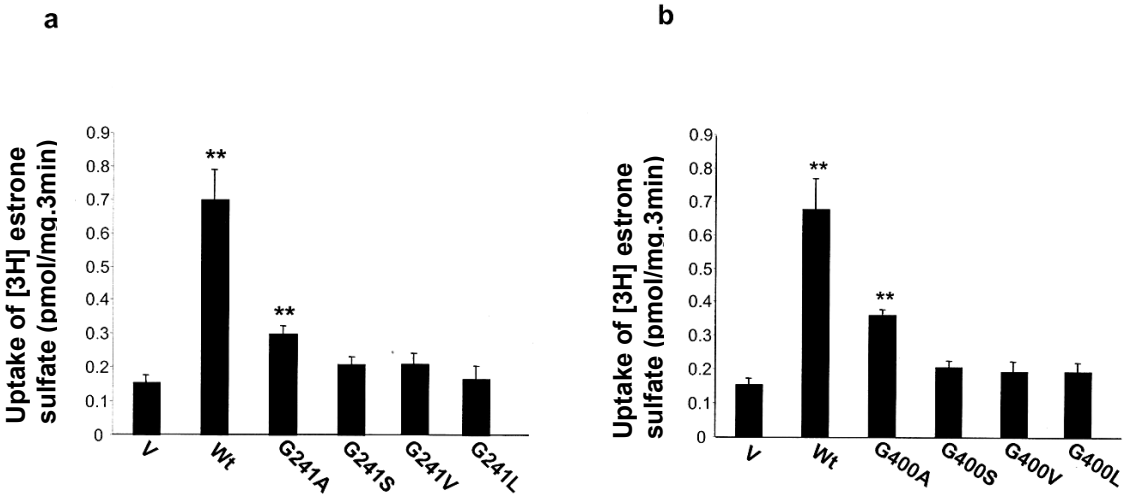

Fig 6. The effects of glycine mutations on hOAT4 function.

Glycine (G) at positions 241 and 400 was individually mutagenized to alanine (A), valine (V), and leucine (L), and the uptake of 100 nM [^{3}H] estrone sulfate in mutant-transfected cells was measured. Mutants G241S and G400S were also included in this study. V, pcDNA vector. Values were mean ± S.E. (*n* = 3). **, *P* < 0.01 versus control.

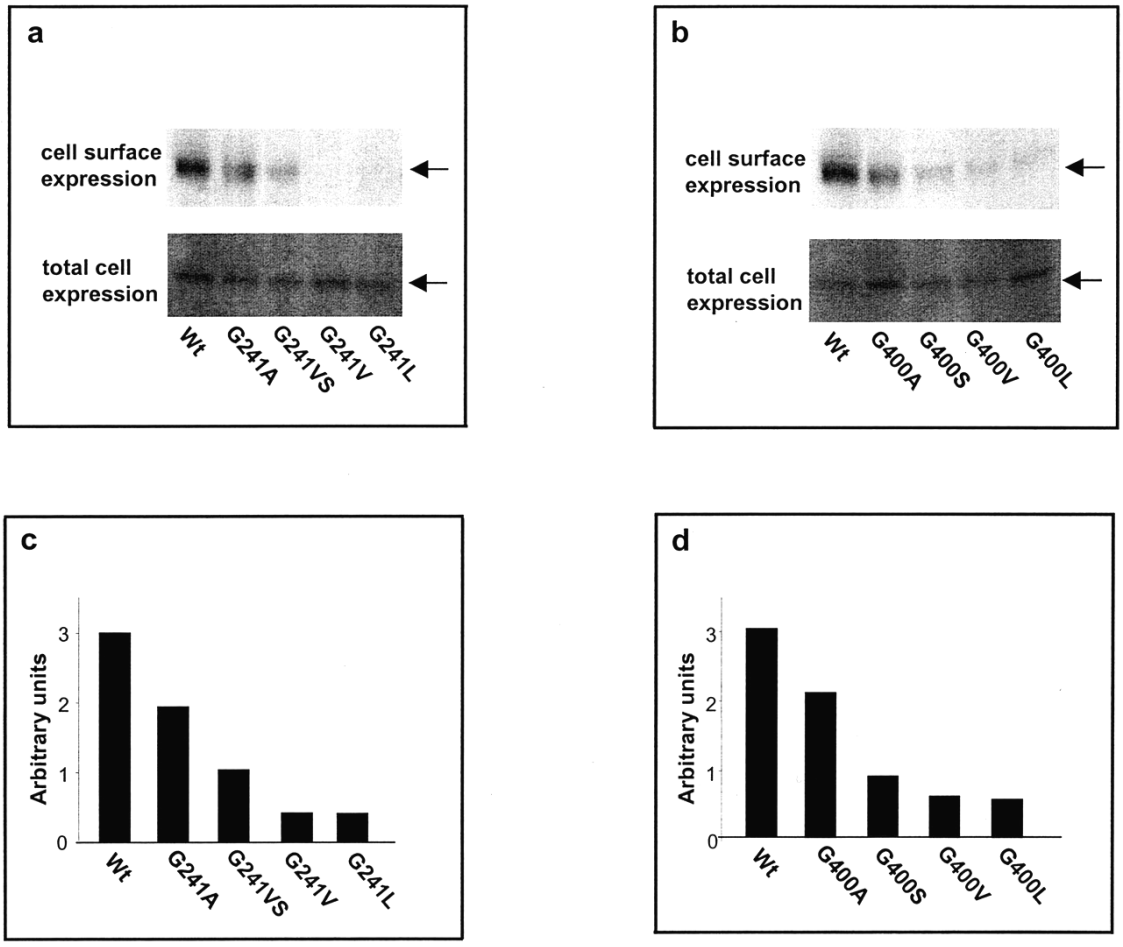

Fig 7. Total cell and surface expression of hOAT4 and its mutants.

For total cell expression (a and b, bottom), COS-7 cells expressing hOAT4 wild type (Wt) and its mutants (G241A, G241S, G241L, G241V, G400A, G400S, G400L, and G400V) were lysed, and their proteins were separated by SDS-PAGE and visualized by immunoblot analysis using anti-hOAT4 antibody. For cell-surface expression (a and b, top), cells expressing hOAT4 wild type (Wt) and its mutants (G241A, G241S, G241L, G241V, G400A, G400S, G400L, and G400V) were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads, separated, and visualized as above. c and d, densitometry analyses of the intensities of the surface expression in a and b, respectively.

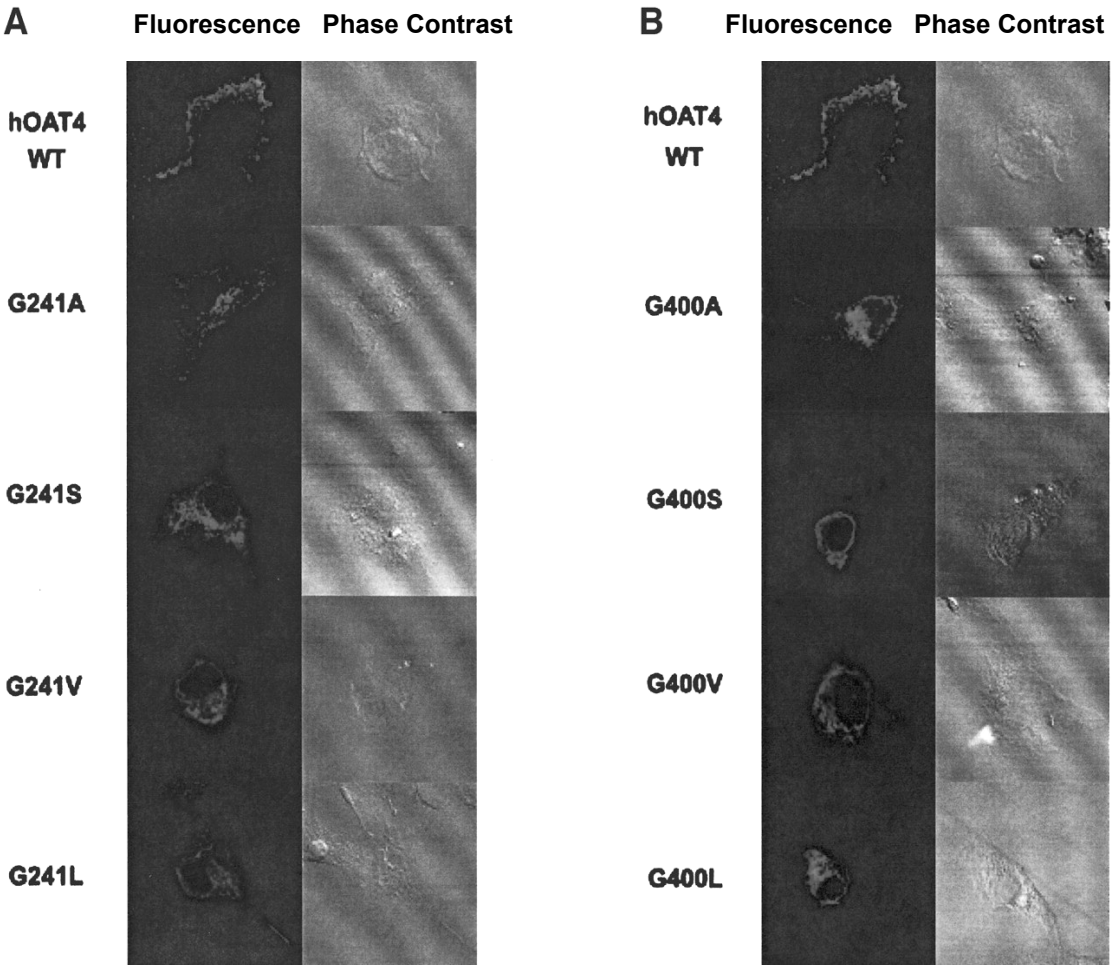

Fig 8. Immunofluorescence study of the effect of substitution of Gly-241 (a) and Gly-400 (b) with larger side chains on the cellular expression of hOAT4.

\Wild-type hOAT4 and its mutants were expressed in COS-7 cells. The cells were then stained with anti-hOAT4 antibody and fluorescein isothiocynate-coupled goat anti-mouse IgG. Specific immunostaining appears as bright fluorescence. Phase contrast images showed that cells were fully attached to the culture dishes under all conditions.

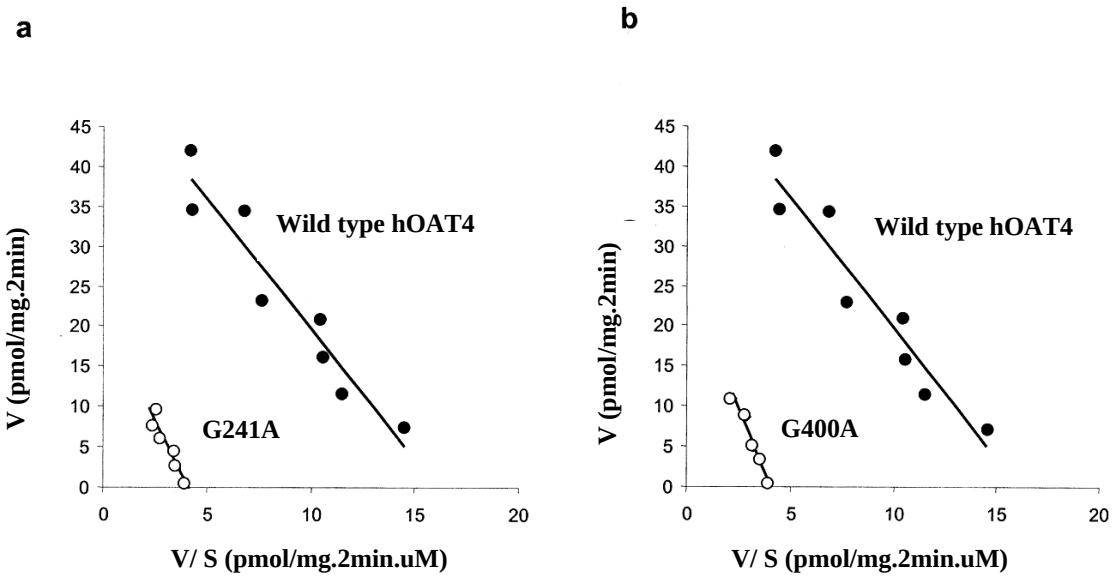

Fig 9. Kinetic analysis of estrone sulfate transport mediated by mutant G400A.

Kinetic characteristics were determined at substrate concentrations ranging from 0.1 to 10 μM (2-min uptake) using cells expressing hOAT4 wild type (Wt; closed circles; a and b), mutant G241A (open circles; a), and mutant G400A (open circles; b). Transport kinetic values were calculated using the Eadie-Hofstee transformation. Values were mean ± S.E. (*n* = 3).

Chapter 3:

Mutational analysis of histidine residues in human organic anion transporter 4 (hOAT4)

This chapter was written in the style of and published in Biochemical Journal (Zhou F, Pan Z, Ma J, You G. Biochem J. 2004 Nov 15; 384 (Pt 1):87-92.)

Abstract

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, antitumor drugs, antibiotics, anti-hypertensives and anti-inflammatories. hOAT4-mediated transport of the organic anion estrone sulphate in COS-7 cells was inhibited by the histidine-modifying reagent DEPC (diethyl pyrocarbonate). Therefore the role of histidine residues in the function of hOAT4 was examined by site-directed mutagenesis. All five histidine residues of hOAT4 were converted into alanine, singly or in combination. Single replacement of His-47 or simultaneous replacement of His-47/52/83 or His-47/52/83/305/469 (H-less) led to a 50–80% decrease in transport activity. The decreased transport activity of these mutants was correlated with a decreased amount of cell-surface expression, although the total cell expression of these mutants was similar to that of wild-type hOAT4. These results suggest that mutation at positions 47, 47/52/83 and 47/52/83/305/469 impaired membrane expression rather than function. We also showed that, although most of the histidine mutants of hOAT4 were sensitive to inhibition by DEPC, H469A (His-469→Ala) was completely insensitive to inhibition by this reagent. Therefore modification of His-469 is responsible for the inhibition of hOAT4 by DEPC.

Keywords

COS-7 cell, diethyl pyrocarbonate (DEPC), histidine, human organic anion transporter 4 (hOAT4), mutagenesis, oestrone sulphate

Abbreviations

DEPC, diethyl pyrocarbonate, ER, endoplasmic reticulum, H-less, mutant with His-47/52/83/305/469 all replaced by alanine, hOAT4, human organic anion transporter-4, NHS-SS-biotin, biotin disulphide *N*-hydroxysuccinimide ester, OAT, organic anion transporter, PBS/CM, PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, Wt, wild-type

Introduction

Organic anion transporters (OATs) play essential roles in the body disposition of clinically important anionic drugs, including anti-HIV therapeutics, antitumor drugs, antibiotics, anti-hypertensives and anti-inflammatories [1,2]. Four OAT isoforms have been identified by us and others [1,2]. OAT1 and OAT3 are predominantly expressed in the kidney and the brain. OAT4 is present mainly in the kidney and the placenta. OAT2 is expressed in the liver.

The plasma membrane of kidney proximal tubule cells is divided into a brush-border membrane, which faces the lumen/urine and a basolateral membrane, which is in contact with the blood. These two membrane domains are functionally and morphologically distinct with different lipid and protein compositions [3]. OAT1 and OAT3 have been localized to the basolateral membrane, where they are responsible for moving organic anion drugs across the basolateral membrane into the proximal tubule cells for subsequent exit/elimination across apical membrane into urine [1,2]. OAT4 has been localized to the brush-border membrane where it is responsible for the reabsorption of organic anion drugs into the proximal tubule cells [4]. These transporters are multispecific with a wide range of substrate recognition. OAT4 interacts with sulphate-conjugated steroids, antibiotics and Ochratoxin A [1,2].

A study using brush-border membrane vesicles from dog kidney [5] showed that histidine-modifying reagents, such as DEPC (diethyl pyrocarbonate) inhibited OAT function. This implied that histidine residues might be critical for organic

anion transport. Based on this previous observation, we predicted that the histidine residues in hOAT4 (human OAT4) might play important roles in its function. The present work was undertaken to examine this hypothesis using chemical modification and site-directed mutagenesis approaches in conjunction with functional assay.

Materials and Methods

Materials

DEPC was purchased from Sigma (St. Louis, MO, U.S.A.). [^{3}H] estrone sulphate was from NEN Life Science Products (Hercules, CA, U.S.A.). NHS-SS-biotin (biotin disulphide *N*-hydroxysuccinimide ester) and streptavidin–agarose beads were purchased from Pierce (Rockford, IL, U.S.A.).

Site-directed mutagenesis

Mutant transporters were generated by site-directed mutagenesis of histidine to alanine of hOAT4. The mutant sequences were confirmed by the dideoxy chain termination method.

Expression in COS-7 cells

COS-7 cells were grown at 37 °C under 5% CO_2 in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, CA, U.S.A.) supplemented with 10% (v/v) fetal bovine serum. Confluent COS-7 cells were transfected with DNA plasmid using LIPOFECTAMINE 2000 reagent (Invitrogen) following the manufacturer's instructions. Transfected cells were incubated for 14–20 h at 37 °C, and then used for transport assay and Western blot analysis.

Treatment with DEPC

COS-7 cell monolayers were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.3) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM). Monolayers were then incubated with DEPC (for 10 min) at the stated concentrations at 23 °C, and then washed four times before isotopic transport measurements.

Transport measurements

For each well, uptake solution was added. The uptake solution consisted of PBS/CM and [^{3}H] estrone sulphate. At the times indicated in the Figure legends, aspirating the uptake solution and rapidly washing the well with ice-cold PBS stopped uptake. The cells were then solubilized in 0.2 M NaOH, neutralized in 0.2 M HCl, and divided into aliquots for liquid-scintillation counting. Uptake count was standardized by the amount of protein in each well. Values were means±S.E.M. (*n*=3).

Cell-surface biotinylation

Cell-surface-expression levels of hOAT4 were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin. The transporters were expressed in COS-7 cells in six-well plates using LIPOFECTAMINE 2000 reagent as described above. After 20 h, the medium was removed, and the cells were washed twice with 3 ml of ice-cold PBS/CM (pH 8.0). The plates were kept on ice and all solutions were ice-cold for the rest of the procedures. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20 min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine, then incubated with the same solution for 20 min on ice to ensure complete quenching of unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 µl of lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 and protease inhibitors: 200 µg/ml PMSF and 3 µg/ml leupeptin), pH 7.4. The unlysed

cells were removed by centrifugation at 15700 g at 4 °C. A 50 µl volume of streptavidin–agarose beads was then added to the supernatant to isolate cell-membrane protein. PAGE and immunoblotting using an anti-hOAT4 antibody detected hOAT4 in the pool of surface proteins.

Electrophoresis and immunoblotting

Protein samples (equal amounts) were resolved on SDS/7.5% PAGE minigels and electroblotted on to PVDF membranes. The blots were blocked for 1 h with 5% (w/v) non-fat dried milk in PBS/0.05% Tween, washed, and incubated for 1 h at 23 °C with monoclonal anti-hOAT4 antibody (1:1000 dilution). The membranes were washed and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:20000 dilution), and signals were detected by SuperSignal West Dura Extended Duration Substrate kit (Pierce).

Immunofluorescence of transfected cells

At 16 h after transfection, COS-7 cells were washed three times in PBS, fixed for 15 min at room temperature (23 °C) in 4% (w/v) Para formaldehyde in PBS, and rewashed in PBS. The fixed cells were then permeabilized with 0.1% Triton X-100 for 10 min. After that, the cells were incubated for 15 min at room temperature in PBS containing 5% (v/v) goat serum and then incubated for 1 h in the same medium containing anti-hOAT4 antibody (3 µg/ml) at room temperature. The cells were washed, and bound primary antibodies were detected by reaction with FITC-coupled goat anti-rabbit IgG (Chemicon International, Temecula, CA, U.S.A.), diluted 1:200 for 1 h. Cells were washed thoroughly, and the cover glasses were mounted in Gel/Mount (Biomeda, Foster City, CA, U.S.A.). Samples were

examined using a Zeiss LSM-510 laser scanning microscope (Carl Zeiss, Thornwood, NY, U.S.A.).

Statistics

To test the significance of differences between data sets, Student's *t* test was performed.

Results

Effects of DEPC on hOAT4 function

A previous study using brush-border membrane vesicles from dog kidney [5] indicated that the OAT system contains functionally important histidine residues that are sensitive to inhibition by histidine-modifying reagents such as DEPC. The cloned hOAT4 expressed in COS-7 cells is also sensitive to inhibition by DEPC. As shown in Figure 1, pre-treatment of hOAT4-expressing cells with DEPC led to a concentration-dependent decrease in hOAT4-mediated transport of [^{3}H] estrone sulphate. Approx. 50% inhibition was reached with 0.2 mM DEPC. This result is consistent with previous observations [5].

Histidine→alanine mutations in hOAT4

To determine whether histidine residues are involved in the transport of oestrone sulphate by hOAT4, site-directed mutagenesis was performed to change all five histidine residues to alanine, singly or in combination. The secondary-structure model of hOAT4, indicating the positions of the five histidine residues, is shown in Figure 2.

Analysis of the effect of single replacement of histidine residues

Oestrone sulphate transport was measured in COS-7 cells transfected with cDNAs for wild-type (Wt) hOAT4 and its histidine mutants with single replacement. As shown in Figure 3, most mutants showed little change in oestrone sulphate transport compared with the Wt control. Mutant H47A (His-47→Ala) exhibited 50% reduction in transport activity. This reduced transport activity could be caused by changes in the absolute number of transporters, turnover rate,

substrate binding affinity or a combination of these factors. As a first step in evaluating possible changes, we compared the protein expression levels of Wt hOAT4 and its mutants in the total cell extracts and on the cell surface by immunoblot analysis (Figure 4). In total cell extracts (lower panel of Figure 4a), the abundance of all the hOAT4 mutants is similar to that of Wt protein, suggesting that similar amounts of the Wt and the mutant proteins are expressed in these cells. In contrast, the abundance of mutant H47A expressed at the cell surface was much lower than that of the Wt (upper panel of Figure 4a). These results indicate that mutation at His-47 impaired the proper targeting of hOAT4 to the plasma membrane. When transport activities of Wt hOAT4 and its mutants (Figure 3) were normalized to the levels of cell-surface expression (upper panel of Figure 4a), the transport efficiencies of all the mutants were similar to that of Wt hOAT4 (Figure 4b).

Analysis of the effect of multiple replacements of histidine residues

Estrone sulphate transport was then measured in COS-7 cells transfected with cDNAs for Wt hOAT4 and its histidine mutants with multiple replacements (Figure 5). A mutant with multiple replacements at the N-terminus (H47/52/83A) exhibited approx. 50% transport activity compared with that of Wt hOAT4. A mutant with all five histidine residues replaced (H-less) exhibited only about 18% transport activity compared with that of Wt hOAT4. Western blot analysis of protein expression in total cell extracts (lower panel of Figure 6a) and at the cell surface (upper panel of Figure 6a) showed that, despite the similar amount of expression among the Wt and its mutants in total cell extracts, the cell-surface expression of mutants

H47/52/83A and H-less was much lower than that of the Wt. These results indicate that mutation at His-47/52/83 and His-47/52/83/305/469 impaired the proper targeting of hOAT4 to the plasma membrane. When transport activities (Figure 5) were normalized to the level of cell-surface expression (upper panel of Figure 6a), the transport efficiencies of mutant H47/52/83A and H-less were similar to that of Wt hOAT4 (Figure 6b).

Immunofluorescence analysis of the expression of histidine mutants

Further evidence of the difficulty of mutant proteins (H47A, H47/52/83A and H-less) to be transported to the plasma membrane was obtained by immunofluorescence. As shown in Figure 7, although the plasma membrane was clearly labeled (shown as bright fluorescence) in cells transfected with Wt hOAT4 and most of its histidine mutants, fluorescence remained mainly in the intracellular compartment in cells transfected with the H-less mutant. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

Effect of DEPC on histidine mutants of hOAT4

The sensitivity of the histidine mutants to the inhibition by DEPC was tested. As shown in Figure 8, while most of the histidine mutants were sensitive to the inhibition by DEPC, H469A was completely insensitive to the inhibition by this reagent. Therefore His-469 represents the binding site for DEPC in hOAT4.

Discussion

Inhibition of OAT activity by histidine-modifying reagents was observed by us [6] and others [5] in COS-7 cells expressing a mouse OAT (mOAT1) and in brush-border membrane vesicles from dog kidney. These studies led to the hypothesis that critical histidine residues are involved in OAT function. In the present study, we tested our hypothesis in hOAT4. All five histidine residues in hOAT4 were replaced by alanine, singly or in combination. We showed that single replacement of His-47 with alanine resulted in a 50% reduction of oestrone sulphate transport, whereas single replacement of histidine residues at other sites had no significant effect on transport function, suggesting that no individual histidine residue is essential for hOAT4 function.

The effect of multiple mutations at the various regions of hOAT4 on transport function was also examined. Replacement of histidine residues at the N-terminus of hOAT4 (H47/52/83A) resulted in a 50% reduction of estrone sulphate transport, whereas replacement of all five histidine residues (H-less) resulted in an 82% decrease in oestrone sulphate transport.

There are several possible mechanisms that could contribute to the reduced transport activity of the histidine mutants (H47A, H47/52/83A and H-less). For example, mutation at these positions may cause the transporter to misfold and be degraded at the ER (endoplasmic reticulum) without reaching the cell surface, a ‘quality-control’ mechanism in the ER. Another possibility is that the mutation may impair the ability of the transporter to target to the cell membrane. Finally, the

mutation may decrease the affinities of the transporter for its substrates. By measuring both total cell and cell-surface expression of these mutants directly, we showed that, despite the similar total cell expression of these mutants relative to that of Wt hOAT4, the surface expression of these mutants was significantly decreased. These results suggest that substitutions of His-47, His-47/52/83 and His-47/52/83/305/469 with alanine do not interfere with the total cell expression of the transporter protein, but rather interfere with the trafficking of the transporter to the plasma membrane. Our immunofluorescence study confirmed these results. The involvement of histidine residues in cell-surface targeting has been reported previously for such membrane proteins as melibiose permease [7] and Na^+/dicarboxylate co-transporter [8].

We also showed in the present study that, although most of the histidine mutants of hOAT4 were sensitive to the inhibition by DEPC, H469A lost sensitivity to the inhibition by this reagent completely, suggesting that the modification of His-469 is responsible for the inhibition of hOAT4 function by DEPC.

In conclusion, we demonstrate that (i) none of the individual histidine residues in hOAT4 is required for function, (ii) multiple histidine residues may play a synergistic role for the targeting of the transporter onto the cell surface, and (iii) the regulation of the transport function by histidine modification occurs through direct modification of His-469.

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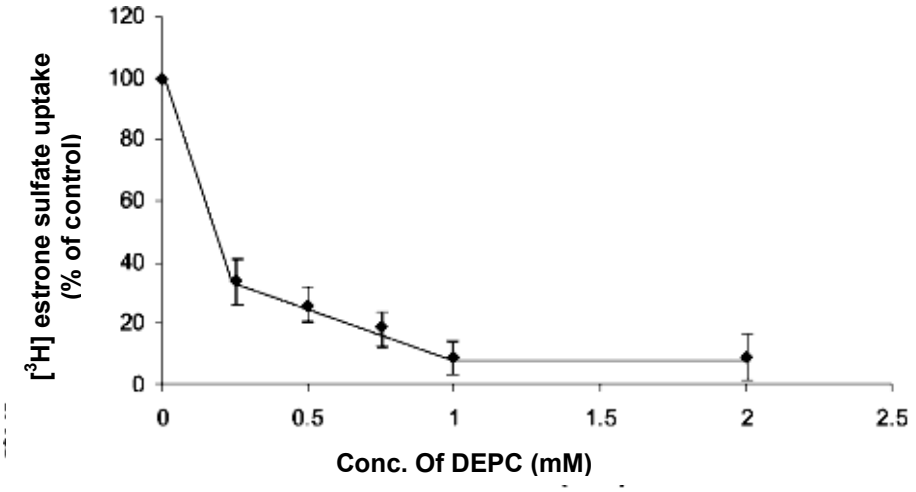

Figure 1 Dose–response of DEPC inhibition

COS-7 cells expressing hOAT4 were pre-incubated with concentrations of DEPC up to 2 mM for 10 min. The solutions were washed away and uptake of 100 nM [^{3}H] estrone sulphate was measured for a 10 min time period. The results shown are means±S.E.M. (*n*=3).

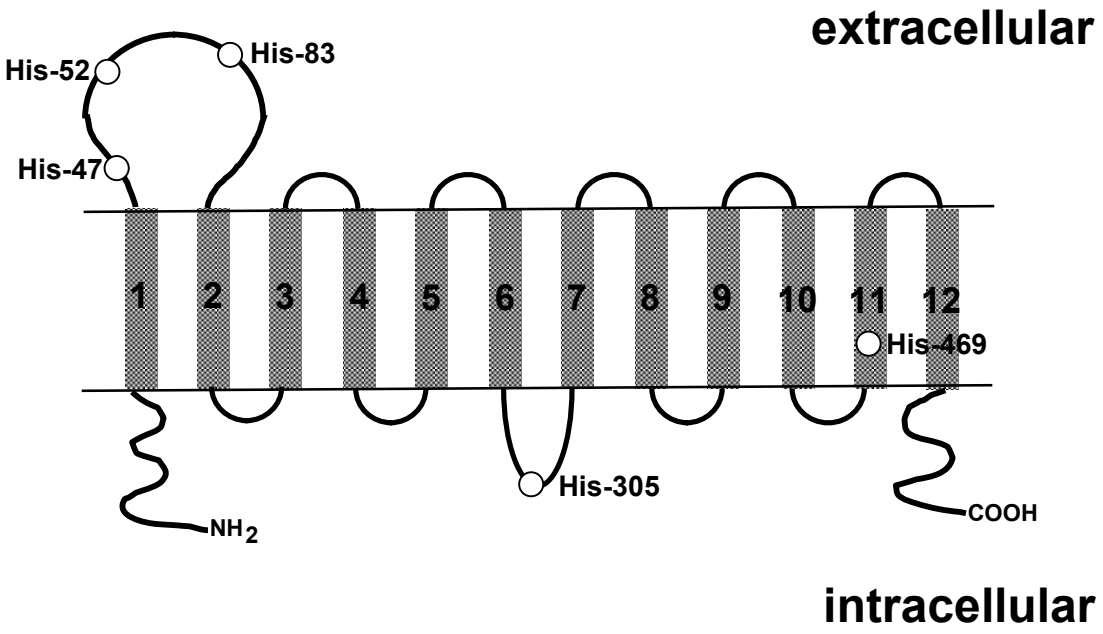

Figure 2 Secondary-structure model of hOAT4

The transmembrane domains are numbered 1–12. The positions of the five histidine residues are shown as open circles and are numbered.

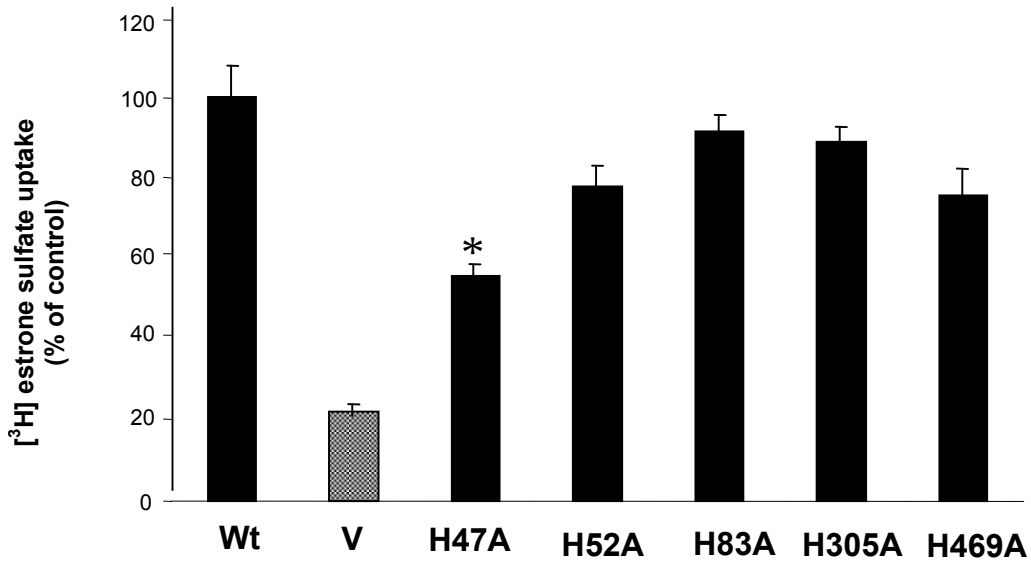

Figure 3 Functional analysis of the effect of single replacement of histidine residues

Cells were transfected with cDNAs for Wt hOAT4, pcDNA vector (mock control, V) and its histidine mutants. At 16 h after the transfection, [^{3}H] estrone sulphate uptake (100 nM) was measured. Results are means±S.E.M. relative to Wt (*n*=3). **P*<0.01 compared with the Wt.

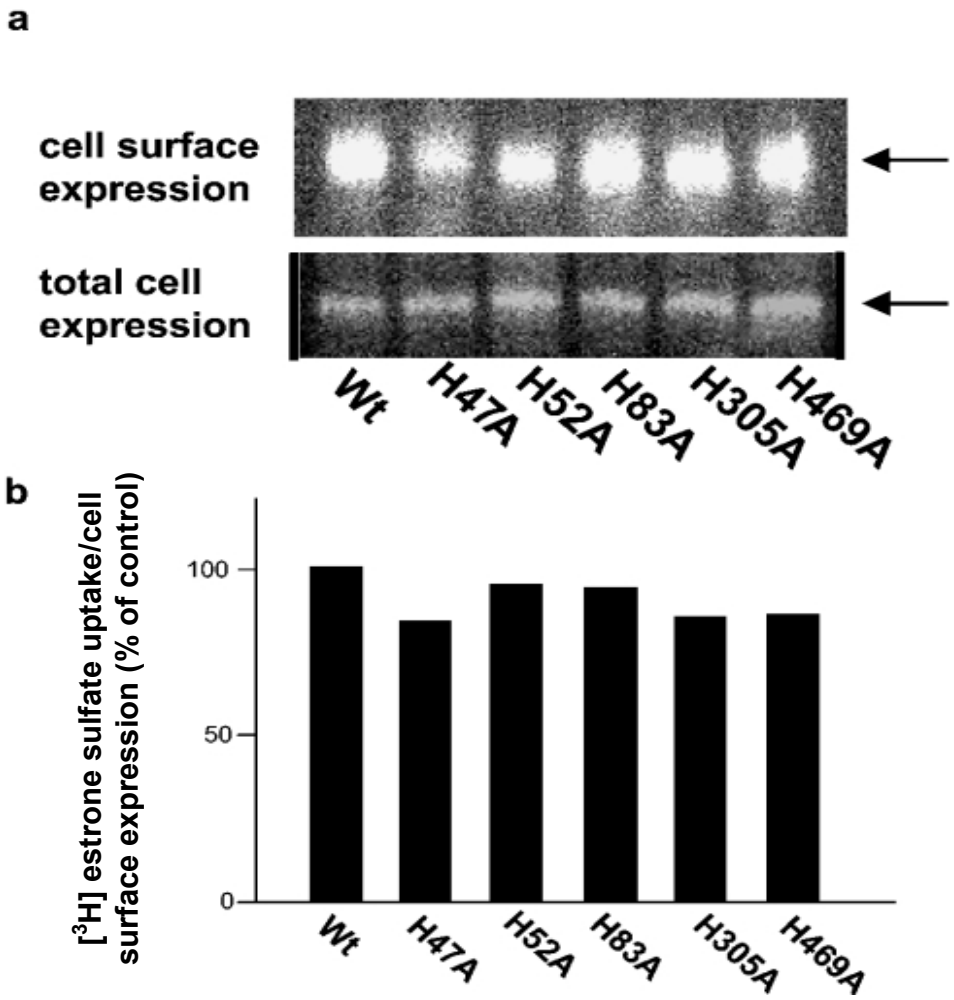

Figure 4 Western blot analysis of the effect of single replacement of histidine residues

(**a**) For cell-surface expression (upper panel), cells expressing Wt hOAT4 and its mutants (H47A, H52A, H83A, H305A and H469A) were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads, separated by SDS/PAGE and visualized by immunoblot analysis using anti-hOAT4 antibody. For total cell expression (lower panel), cells expressing Wt hOAT4 and its mutants (H47A, H52A, H83A, H305A and H469A) were lysed, and their proteins were separated and visualized as above.

(**b**) The transport activities of Wt hOAT4 and its mutants were normalized to their respective cell-surface expression.

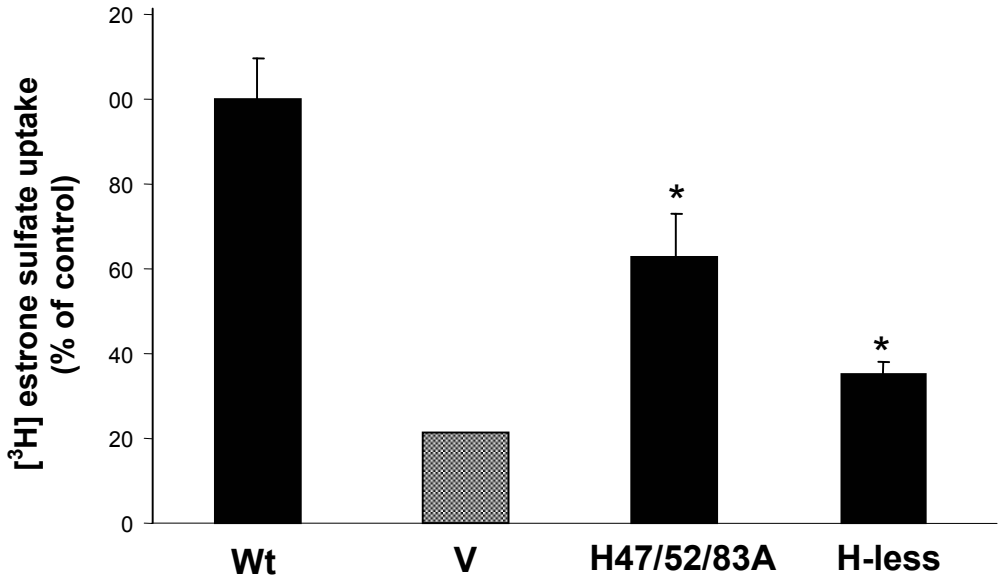

Figure 5 Functional analysis of the effect of multiple replacements of histidine residues

Cells were transfected with cDNAs for Wt hOAT4, pcDNA vector (mock control, V) and its histidine mutants (H/47/52/83 and H-less). At 16 h after transfection, [^{3}H] estrone sulphate uptake (100 nM) was measured. Results are means±S.E.M. relative to the Wt. *P<0.01 compared with Wt.

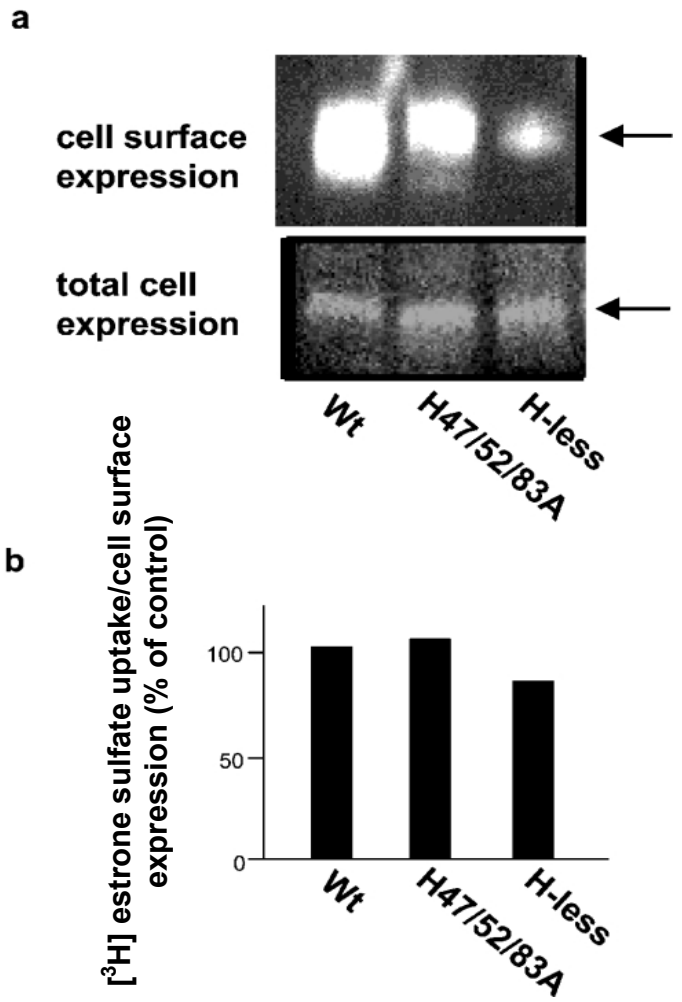

Figure 6 Western blot analysis of the effect of multiple replacements of histidine residues

(a) For cell-surface expression (upper panel), cells expressing Wt hOAT4 and its mutants (H47/52/83A and H-less) were biotinylated, and the labeled cell-surface proteins were precipitated with streptavidin beads, separated by SDS/PAGE and visualized by immunoblot analysis using anti-hOAT4 antibody. For total cell expression (lower panel), cells expressing Wt hOAT4 and its mutants (H47/52/83A and H-less) were lysed, and their proteins were separated and visualized as above.

(b) The transport activities of Wt hOAT4 and its mutants were normalized to their respective cell-surface expression.

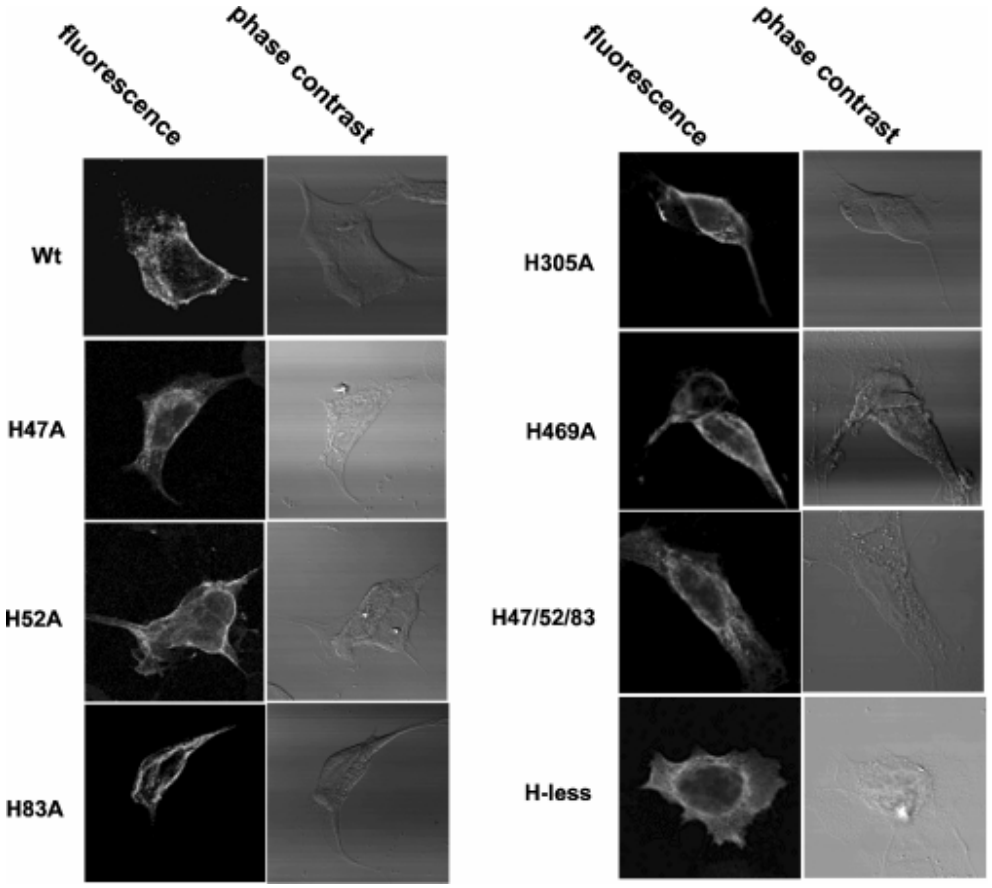

Figure 7 Immunofluorescence study of the effect of replacement of histidine residues on the cellular expression of hOAT4

Wt hOAT4 and its histidine mutants were expressed in COS-7 cells. The cells were then stained with anti-hOAT4 antibody and FITC-coupled goat anti-rabbit IgG. Specific immunostaining appears as bright fluorescence. Phase-contrast images showed that cells were fully attached to the culture dishes under all conditions.

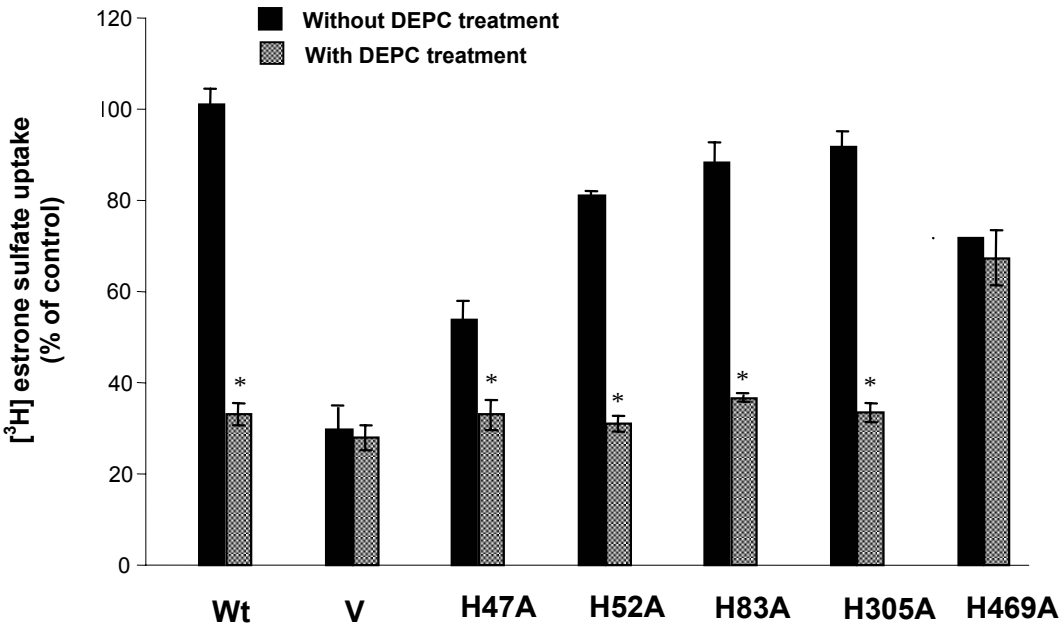

Figure 8 Effects of DEPC on histidine mutants of hOAT4

COS-7 cells, expressing Wt hOAT4, pcDNA vector (mock control, V) and its mutants, were treated with (hatched bar) or without (black bar) 0.75 mM DEPC, followed by measuring uptake of 100 nM [^{3}H] estrone sulphate. Results are means±S.E.M. relative to Wt. *P<0.01 compared with respective values obtained in the absence of DEPC.

Chapter 4:

Regulation of human organic anion transporter 4 (hOAT4) by progesterone and protein kinase C in human placental BeWo cells.

This chapter was written in the style of and published in American journal of physiology. Endocrinology and metabolism (Zhou F, Hong M, You G. Am J Physiol Endocrinol Metab. 2007 Mar 6)

Abstract

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories. hOAT4 is abundantly expressed in the placenta. In the current study, we examined the regulation of hOAT4 by pregnancy-specific hormones progesterone (P4) and 17?-estradiol (E2) and by protein kinase C (PKC) in human placental BeWo cells. P4 induced a time- and concentration-dependent down regulation of hOAT4 transport activity, whereas E2 had no effect on hOAT4 function. The down regulation of hOAT4 activity by P4 mainly resulted from a decreased cell surface expression without a change in total cell expression of the transporter, kinetically revealed as a decreased Vmax without significant change in Km. Activation of PKC by phorbol 12,13-dibutyrate (PDBu) also resulted in an inhibition of hOAT4 activity through a decreased cell surface expression of the transporter. However, P4-induced down regulation of hOAT4 activity could not be prevented by treating hOAT4-expressing cells with PKC inhibitor, staurosporine. We concluded that both P4 and activation of PKC inhibited hOAT4 activity through redistribution of the transporter from cell surface to the intracellular compartments. However, P4 regulates hOAT4 activity by mechanisms independent of PKC pathway.

Introduction

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories (3, 7, 18, 19, 24). hOAT4 is abundantly expressed in the kidney and placenta (4). In the kidney, OAT4 functions as an organic anion/dicarboxylate exchanger at the apical membrane of the proximal tubule and is responsible for the reabsorption of organic anions driven by an outwardly directed dicarboxylate gradient (6). In the placenta, hOAT4 is localized to the basolateral membrane of syncytiotrophoblasts (20). It is believed that estrogen biosynthesis in the placenta uses dehydroepiandrosterone-sulfate (DHEAS), a precursor produced in large amount by the fetal adrenals. Accumulation of excess DHEAS is associated with intrauterine growth retardation (IUGR) (17). DHEAS is an OAT4 substrate. Therefore, OAT4 may play an important role in efficient uptake of DHEAS by the placenta for the production of estrogens and for the protection of fetus from the cytotoxicity of DHEAS. Computer modeling showed that hOAT4 contains multiple potential glycosylation sites in its first extracellular loop. Glycosylation process occurs in two major steps: first step is the addition of oligosaccharides to the nascent protein and the second step is the processing of added oligosaccharides, in which, the added oligosaccharides are modified and trimmed. We previously showed (27) that addition of oligosaccharides but not the processing of the added oligosaccharides plays a critical role in the targeting of hOAT4 to the plasma membrane. Processing

of added oligosaccharides may not be essential in determining the substrate spectra of hOAT4 and its property as an organic anion exchanger. However, the processing of added oligosaccharides from mannose rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates.

To date, little is known about how hOAT4 is regulated in the placenta. Progesterone (P4) and 17B-stradiol (E2) are the two most important steroid hormones produced by human placenta during pregnancy. These hormones have been shown to affect the expression and activities of other placental transporters (22). In the present study, we investigated the effects of these hormones on hOAT4 function in human placental BeWo cells, and the possible interaction of these hormones with PKC pathway.

Materials and Methods

Materials

[3H] estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL). Progesterone (P4, P-8783), 17?-Estradiol (E2, E-2758), Staurosporine from *Streptomyces* sp. (S-5921), and Phorbol 12,13-dibutyrate (PDBu, P-1269) were purchased from Sigma–Aldrich (St. Louis, MO). Dulbecco's modified Eagle's/F-12 medium (phenol-red free) was purchased from Gibco (Grand Island, NY). Charcoal/dextran-stripped fetal bovine serum was purchased from HyClone (Logan, UT).

Cell culture

Parental BeWo b30-10 cells were grown in Dulbecco's modified Eagle's/F-12 medium (phenol-red free) supplemented with 5% Charcoal/dextran stripped fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO2 atmosphere at 37 °C. BeWo b30-10 cells stably expressing hOAT4 (25) were maintained in Dulbecco's modified Eagle's/F-12 medium (phenol-red free) supplemented with 5% Charcoal/dextran-stripped fetal bovine serum, 0.5 mg/ml geneticin (G418; Invitrogen, Carlsbad, CA), and glucose (100 mg/ml) in a 5% CO2 atmosphere at 37 °C.

Transport measurement

Cells plated in 48-well plates were treated with each reagent at 37 °C for certain time periods as shown in the figure legends. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca2+/Mg2+

(PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 1 mM CaCl2, and 1 mM MgCl2, pH 7.4) and [3H]estrone sulfate. At the times indicated in the figure legends, aspirating off the uptake solution and rapidly washing the well with ice-cold PBS stopped the uptake. The cells were then solubilized in 0.2N NaOH, neutralized in 0.2N HCl, and aliquoted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values were mean ± S.E. (n = 3).

Cell surface biotinylation

Cell surface expression levels of hOAT4 were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Pierce Chemical). The BeWo b30-10 cells stably expressing hOAT4 and parental BeWo b30-10 cells were seeded onto 6-well plate at 8 × 105 per well. After 48 h, the medium was removed, and the cells were washed twice with 3 ml of ice-cold PBS, pH 8.0. The plates were kept on ice, and all solutions were kept ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS containing 100 mM glycine, and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 µl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and protease inhibitors phenylmethylsulfonyl fluoride, 200 µg/ml, and leupeptin, 3µg/ml, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4 °C.

Streptavidin-agarose beads (50 µl; Pierce Chemical) were then added to the supernatant to isolate cell membrane protein. hOAT4 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-hOAT4 antibody (Alpha Diagnostic International, Inc., San Antonio, TX).

Electrophoresis and Western blotting

Protein samples (100 µg) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS/0.05% Tween, washed, and incubated overnight at 4 °C with polyclonal antihOAT4 antibody (1:500, Alpha Diagnostic International, Inc.). The membranes were washed and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000), and signals were detected by SuperSignal West Dura Extended Duration Substrate Kit (Pierce Chemical).

Data analysis

Each experiment was repeated a minimum of three times. The statistical analysis given was from multiple experiments. Statistical analysis was performed using Student's paired t tests. A p value of <0.05 was considered significant.

Results

Effects of P4 and E2 on hOAT4 function.

We first examined whether treatment with P4 or E2 can affect hOAT4 transport activity in BeWo cells. Since the hOAT4 expression vector for the current study does not contain the promoter region of hOAT4, the long-term regulation at the transcriptional level can't be investigated. We only focused on the short-term regulation of the transporter (within a time frame of 1 hr). P4 induced a time- and concentration-dependent inhibition of estrone sulfate uptake (Fig. 1a), whereas E2 had no significant effect on hOAT4 function (Fig. 1b). To further examine the mechanism of P4-induced down-regulation of hOAT4 activity, we determined [3H] estrone sulfate uptake at different substrate concentrations. An Eadie-Hofstee analysis of the derived data (Fig. 2) showed that pre-treatment with P4 resulted in a decreased *V*max (0.154±0.004 pmol/μg.2 min) with untreated cells, and 0.080±0.025 pmol/μg.2 min in the presence of P4) with no significant change in the affinity for estrone sulfate (14.3 ± 0.6 μM with untreated cells and 13.2 ± 0.5 μM in the presence of P4). Determination of the protein concentrations in control cells confirmed that P4 treatment did not change the total protein content of the cultures (data not shown).

Effect of P4 on hOAT4 expression.

A decreased *V*max could be affected by either a reduced number of the transporter at the cell surface or a reduced transporter turnover number (1, 9, 12, 21, 26). To differentiate between these possibilities, we determined transporter expression both at the cell surface and in the total cell lysates. We showed (Fig. 3)

that P4 treatment resulted in a reduced cell surface expression of hOAT4 without affect the total cell expression of the transporter.

Effect of activation of protein kinase C on hOAT4 expression.

We previously showed (25) that activation of PKC by phorbol 12-myristate 13-acetate (PMA) and Phorbol 12,13-dibutyrate (PDBu) led to an inhibition of hOAT4 activity. However, the mechanism underlying such inhibition was not investigated in that study. Here we examined whether such inhibition also, like the effect of P4, result from a decreased cell surface expression of the transporter. Our result showed (Fig. 4) that, indeed, similar to the effect of P4, PDBu treatment also resulted in a reduced cell surface expression of hOAT4 without affect its total cell expression.

Relationship between P4 and PKC.

Both P4 and PKC activator PDBu inhibited hOAT4 activity through a decreased cell surface expression of the transporter. This led us to hypothesize that P4 exerts its effect through the activation of PKC. To test this hypothesis, we treated hOAT4-expressing BeWo cells with P4 in the presence of a PKC inhibitor staurosporine. As shown in Fig. 5, although staurosporine efficiently reversed the inhibitory effect of PDBu on hOAT4 activity as well as its cell surface expression, it could not reverse the inhibitory effect of P4 on hOAT4 activity and its cell surface expression.

Discussion

hOAT4 belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs. hOAT4 is abundantly expressed in the kidney and placenta. Although the regulation of hOAT4 in the kidney has begun to be explored, its regulation in the placenta is largely unknown. The present study investigated the regulation of hOAT4 in human placental BeWo cells by steroid hormones P4 and E2 and by PKC and explored the underlying mechanisms underlying their regulation. We found that P4 acutely inhibited hOAT4 activity at a relatively high concentration 10-5 M (Fig. 1). It was reported that the plasma P4 concentration at term is ~ 0.7 x 10-6 M, and the intracellular P4 concentrations in placenta were about 12-fold higher than those in maternal plasma (11). Therefore, 10-5 M P4 could be reached in the placenta at term. The plasma E2 concentrations are around 10-8 and 10-7 M during pregnancy (2, 11). However, we did not observe any significant effect of E2 on hOAT4 activity within a concentration range of 10-8 M – 10-5 M (Fig. 1). Our kinetic analysis of the inhibition of hOAT4 activity by P4 showed (Fig. 2) that the reduced transport activity was contributed by a reduced maximum transport velocity *V*max without affecting the binding affinity (1/*Km*) for the substrates. *V*max can be affected by either the number of the transporter at the cell surface or the transporter turnover number (1, 9, 12, 21, 26). To differentiate between these possibilities, we determined the effect of P4 on hOAT4 expression both at the cell surface and in the total cell lysates. Our results showed (Fig. 3) that P4 treatment resulted in a reduced cell surface expression of hOAT4 without affecting its total cell expression,

suggesting that a redistribution of hOAT4 from cell surface to the intracellular compartments occurred during such treatment. Such redistribution was observed previously from other membrane transporters (10, 13). In response to stimuli, these transporters were removed from the cell surface to intracellular compartments, where they waited for the next signal to recycle back to the cell surface. An example is the Na+, K+-ATPase (16). Treatment of *Xenopus* oocytes with progesterone resulted in the retrieval of both endogenously expressed and exogenously injected Na+, K+-ATPase from the cell surface. The treatment of progesterone also led to an increased endocytotic activity. Coated pits and vesicles appeared in the oocytes plasma membrane that might be involved in endocytosis, suggesting that progesterone-induced redistribution of Na+, K+-ATPase may occur through endocytotic pathway. Whether such pathway is also involved in progesterone-induced rapid redistribution of hOAT4 needs further investigation. The physiological significance of down regulation of hOAT4 function by P4 remains speculative. ABCG2, also called breast cancer resistance protein and an efflux pump for various compounds in placenta, was also shown to be down regulated by P4 in placental BeWo cells (23). Several studies (8, 14, 15) reported a gestational age-dependent decrease in the expression of p-glycoprotein (P-gp), suggesting that placental P-gp expression is under developmental control. Considering the role as an efflux pump for xenobiotics, the gestational age dependent expression of P-gp in placenta makes teleological sense. The fetus is at greatest danger to toxic insult from xenobiotics early in pregnancy. Therefore up-regulation of the expression of P-gp early in pregnancy is a mechanism used to

protect the fetus from toxicological insult. It is known that progesterone concentration increases with gestational age. This led us to hypothesize that hOAT4 expression in placenta may also be developmentally regulated with highest expression in early pregnancy. We are currently testing such hypothesis. In a previous study, we showed (25) that activation of PKC by PDBu led to an inhibition of hOAT4 activity in BeWo cells. However, the mechanism underlying such inhibition was not investigated. In the present study, we showed (Fig. 4) that, PDBu treatment resulted in a reduced cell surface expression of hOAT4 without affect its total cell expression, suggesting that like the effect of P4, PDBu also caused a redistribution of hOAT4 from cell surface to the intracellular compartments. Because both P4 and PDBu induced redistribution of hOAT4, we then asked whether P4 inhibited hOAT4 activity through activation of PKC. It was indicated that P4 may exert its effect through intracellular mechanisms dependent on PKC (5). However, our results showed (Fig. 5) that pre-treating the cells with PKC inhibitor staurosporine could not prevent the inhibitory effect of P4. In conclusion, we are first to show that both P4 and activation of PKC inhibited hOAT4 activity through redistribution of the transporter from cell surface to the intracellular compartments. However, P4 regulates hOAT4 activity by mechanisms independent of PKC pathway.

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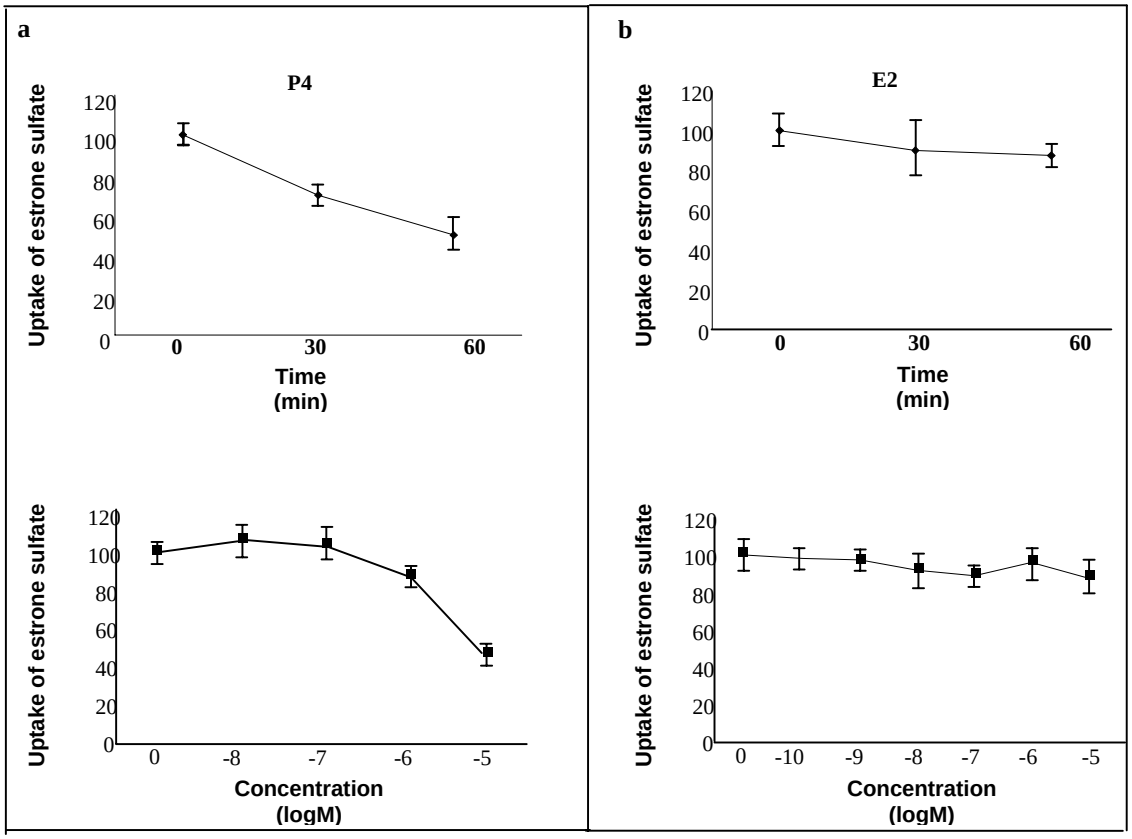

Fig 1. Effect of P4 and E2 on hOAT4 activity in BeWo cells.

hOAT4-expressing BeWo b30-10 cells were treated with P4 (a) or E2 (b) at various concentrations for 30 min and 1 hr followed by [3H] estrone sulfate uptake (4 min, 100 nM). Uptake activity was expressed as a percentage of the uptake measured in untreated cells. The results represent data from three experiments. The uptake values in mock cells (parental BeWo b30-10 cells) were subtracted. Values are mean±S.E. (*n* = 3).

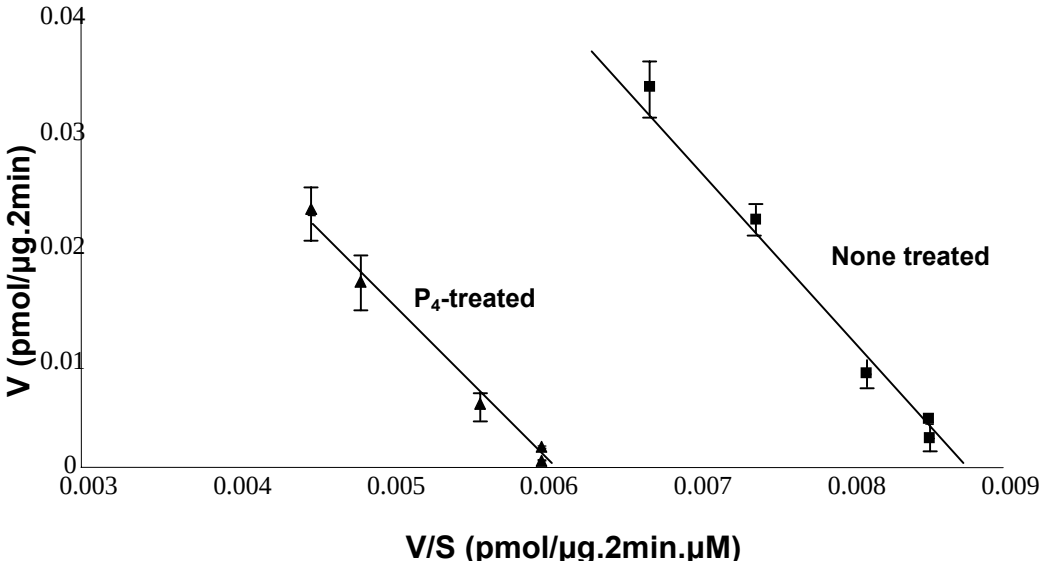

Fig 2. Effect of P4 on the kinetics of estrone sulfate transport.

BeWo b30-10 Cells expressing hOAT4 were pre-treated with or without P4 (10-5 M) for 1 hr and initial uptake (2 min) of [3H] estrone sulfate was measured at 0.05–5 μM estrone sulfate. The data represent uptake into hOAT4-transfected cells minus uptake into mock cells (parental BeWo b30-10 cells). Values are mean±S.E. (*n* = 3).

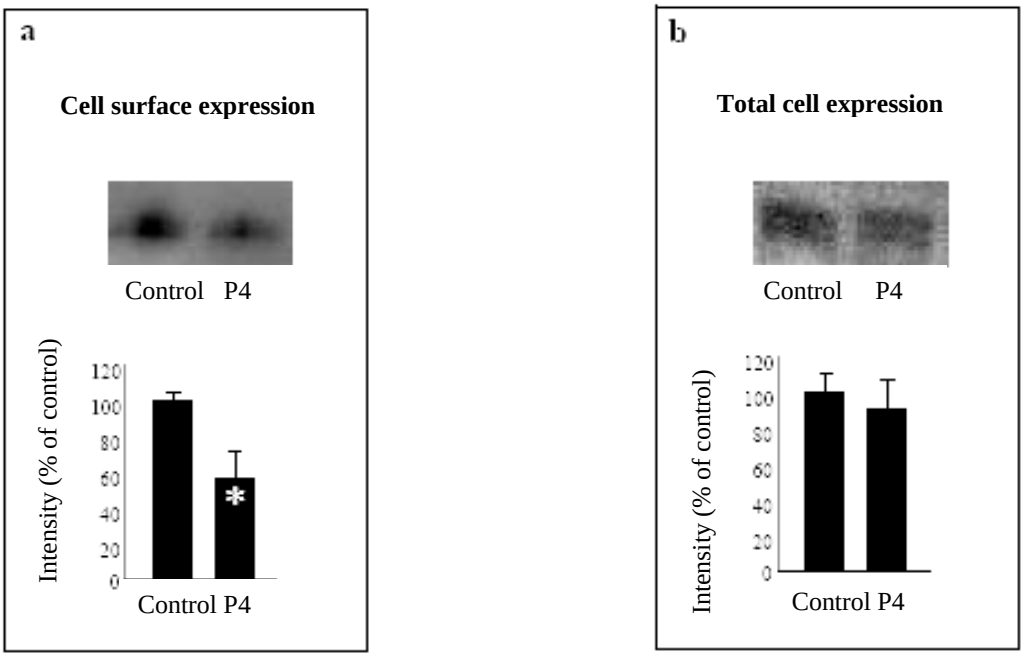

Fig 3. Effect of P4 on cell surface and total cell expression of hOAT4.

a. Western blot analysis of cell surface expression of hOAT4 in cells treated with or without P4 (10-5 M, 1 hr). *Top panel:* BeWo b30-10 Cells stably expressing hOAT4 were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody (1:500). *Bottom panel:* The intensity of the transporter expression from the experiment shown in *top panel* and other experiments was quantified. *Asterisks* indicate values significantly different ($X < 0.05$) from that of untreated cells.

b. Western blot analysis of total cell expression of hOAT4 in cells treated with or without P4 (10-5 M, 1 hr). *Top panel:* Total cell expression of hOAT4 in cells treated with or without P4 (10-5 M, 1 hr). Cells were lysed, and their proteins were separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody. *Bottom panel:* The intensity of the transporter expression from the experiment shown in *top panel* and other experiments was quantified.

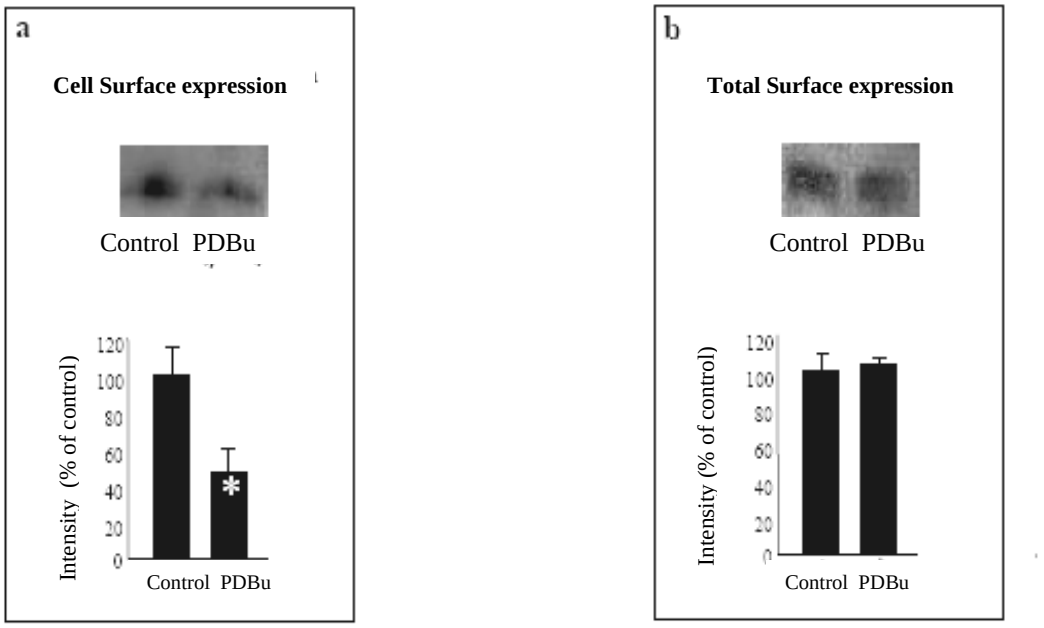

Fig 4. Effect of PDBu on cell surface and total cell expression of hOAT4.

a. Western blot analysis of cell surface expression of hOAT4 in cells treated with or without PDBu (10-6 M, 15 min). *Top panel:* BeWo b30-10 Cells stably expressing hOAT4 were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody (1:500). *Bottom panel:* The intensity of the transporter expression from the experiment shown in *top panel* and other experiments was quantified. *Asterisks* indicate values significantly different ($X < 0.05$) from that of untreated cells.

b. Western blot analysis of total cell expression of hOAT4 in cells treated with or without PDBu (10-6 M, 15 min). *Top panel:* Total cell expression of hOAT4 in cells treated with or without PDBu (10-6 M, 15 min). Cells were lysed, and their proteins were separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody. *Bottom panel:* The intensity of the transporter expression from the experiment shown in *top panel* and other experiments was quantified.

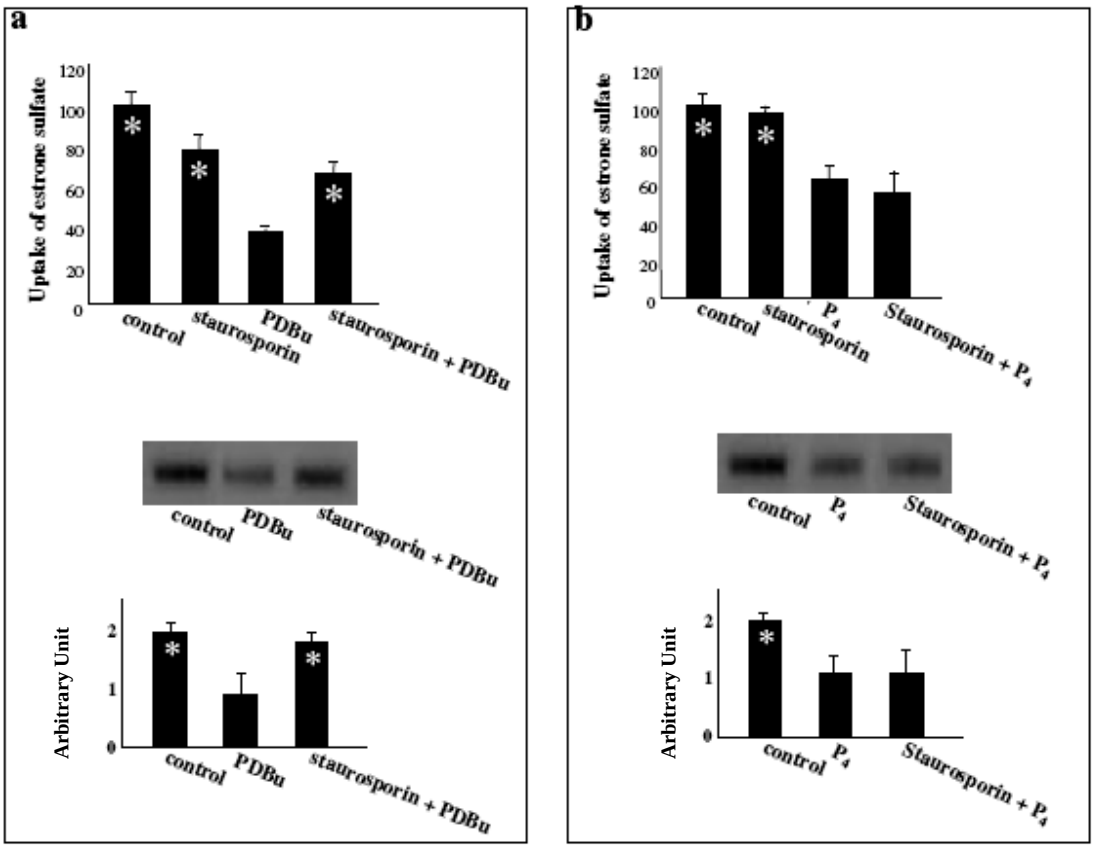

Fig 5. P4-induced inhibition of hOAT4 activity and surface expression is independent of activation of PKC.

a. *Top panel:* Effect of staurosporine on PDBu induced inhibition of hOAT4 activity. BeWo b30-10 Cells stably expressing hOAT4 were pre-treated with PDBu (10-6 M, 15 min) in the presence or absence of staurosporine (2 μM) followed by [3H]estrone sulfate uptake (4 min, 100 nM). *Middle panel:* Effect of staurosporine on PDBu-induced inhibition of hOAT4 surface expression. BeWo b30-10 Cells stably expressing hOAT4 were retreated with PDBu (10-6 M, 15 min) in the presence or absence of staurosporine (2 μM) followed by biotinylation. Labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody (1:500). *Bottom panel:* The intensity of the transporter expression from the experiment

shown in *Middle panel* and other experiments was quantified. *Asterisks* indicate values significantly different (X < 0.05) from that of PDBu-treated cells.

b. *Top panel:* Effect of staurosporine on P4-induced inhibition of hOAT4 activity. hOAT4-expressing cells were pre-treated with P4 (10-5 M, 1 hr) in the presence or absence of staurosporine (2 μM) followed by [3H] estrone sulfate uptake (4 min, 100 nM). The results represent data from three experiments. The uptake values in mock cells (parental BeWo b30-10 cells) were subtracted. Values are mean±S.E. (*n* = 3). *Middle panel:* Effect of staurosporine on P4-induced inhibition of hOAT4 surface expression. BeWo b30-10 Cells stably expressing hOAT4 were pre-treated with P4 (10-5 M, 1 hr) in the presence or absence of staurosporine (2 μM) followed by biotinylation. Labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by Western blotting with anti-hOAT4 antibody (1:500). *Bottom panel:* The intensity of the transporter expression from the experiment shown in *Middle panel* and other experiments was quantified. *Asterisks* indicate values significantly different (X < 0.05) from that of P4-treated cells.

Chapter 5:

Comparison of the Interaction of Human Organic Anion Transporter hOAT4 with PDZ Proteins between Kidney Cells and Placental Cells

This chapter was written in the style of and published in Pharmaceutical Research (Zhou F, Xu W, Tanaka K, You G., Pharm Res. 2007 accepted)

Purpose. To compare the interaction of human organic anion transporter hOAT4 with PDZ proteins between kidney cells and placental cells.

Materials and Methods. PDZ proteins PDZK1 and NHERF1 were transfected into kidney LLC-PK1 cells and placental BeWo cells expressing hOAT4 or hOAT4-Δ, which lacks the PDZ consensus binding site. The interaction of PDZK1 and NHERF1 with hOAT4 and hOAT4- Δ was investigated by measurement of [^{3}H] estrone sulfate uptake, cell surface and total cell expression of hOAT4, and by co-immunoprecipitation experiments.

Results. PDZK1 and NHERF1 enhanced hOAT4 activity in LLC-PK1 cells by increasing the cell surface expression of the transporter. In contrasts, these two PDZ proteins had no effect on hOAT4 activity in BeWo cells.

Conclusion. The interaction of PDZ proteins with hOAT4 may be cell-specific. In placenta, a different set of interacting proteins from PDZK1 and NHERF1 may be required to modulate hOAT4 activity.

Key words. PDZ proteins, Organ-specific interaction, drug transporter, estrone sulfate transport, cell surface biotinylation, co-immunoprecipitation.

Introduction

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories (1-5). The expression of hOAT4 is detected in the kidney and placenta (6). In the kidney, OAT4 functions as an organic anion/dicarboxylate exchanger at the apical membrane of the proximal tubule and is responsible for the reabsorption of organic anions driven by an outwardly directed dicarboxylate gradient (7). In the placenta, hOAT4 is localized to the basolateral membrane of syncytiotrophoblasts (8).

Estrogen biosynthesis in the placenta uses dehydroepiandrosterone-sulfate (DHEAS), a precursor produced in large amount by the fetal adrenals. Accumulation of excess DHEAS is associated with intrauterine growth retardation (IUGR) (9). DHEAS is an OAT4 substrate. Therefore, OAT4 may play an important role in efficient uptake of DHEAS by the placenta for the production of estrogens and for the protection of fetus from the cytotoxicity of DHEAS.

Computer modeling based on hydropathy analysis showed that all the OATs cloned so far contain multiple potential N-linked glycosylation sites in its first extracellular loop. Glycosylation of proteins occurs in two major steps: first step is the addition of oligosaccharides to the nascent protein and the second step is the processing/modification of added oligosaccharides. We previously showed (10) that addition of oligosaccharides but not the processing of the added oligosaccharides plays a critical role in the targeting of hOAT4 to the plasma

membrane. However, the processing of added oligosaccharides from mannose-rich type to complex type is important for enhancing the binding affinity of hOAT4 for its substrates.

hOAT4 contains a PDZ consensus-binding site (S-T-S-L) at its carboxyl terminus. A relatively large number of PDZ proteins have been identified. These PDZ proteins interact with the PDZ consensus-binding site of its target protein and modulate its function. It has been shown that in human embryo kidney HEK293 cells, PDZ proteins PDZK1 and NHERF1 enhanced hOAT4 function through enhancing the surface expression of the transporter (11). PDZK1 and NHERF1 are also expressed in human placenta (11). However, whether the same set of PDZ proteins interact with hOAT4 in placenta is not known. In the current study, we compared the interaction of these PDZ proteins with hOAT4 in kidney LLC-PK1 cells and human placenta BeWo cells.

Materials and Methods

[^{3}H] estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL). LLC-PK1 cells were purchased from American Type Culture Collection (Manassas, VA). BeWo b30-10 cell line was provided by Dr. Nicholas P. Illsley (Robert Wood Johnson Medical School, University of Medicine & Dentistry of New Jersey, Piscataway, NJ). Human PDK1 cDNA (Accession Number: BC006518) and human NHERF1 cDNA (Accession Number: BC011777) are purchased from Open Biosystems (Huntsville, AL). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

Construction of epitope-tagged and mutant transporters

To facilitate immunodetection of hOAT4, epitope tag FLAG was added to N-terminus of hOAT4 (hOAT4-N-flag) by site-directed mutagenesis. Other mutant transporters were generated by site-directed mutagenesis using hOAT4 or hOAT4-N-flag as a template. The mutant sequences were confirmed by the dideoxy chain termination method.

Generation of LLC-PK1 cells stably expressing hOAT4 and its mutants

LLC-PK1 cells were grown in Medium 199, supplemented with 10% fetal calf serum, penicillin/streptomycin (100 units/ml) in a 5% CO_2 atmosphere at 37 °C. Cells were seeded at 3 x 10^6/100-mm dish 24 h before transfection. For transfection of transporter cDNA, a Lipofectamine 2000 reagent was used following manufacture's instruction. After 6 days of selection in medium containing

0.8 mg/ml Geneticin (G418; Invitrogen), resistant colonies were replated to 96 wells for cloning, expansion and analyzing positive clones.

Cell culture and transfections

Parental LLC-PK1 cells were grown in Medium 199 containing 10% fetal bovine serum in a 5% CO2 atmosphere at 37 °C. LLC-PK1 stable cells were grown in Medium with 0.8 mg/ml G418. Parental cells were grown in Dulbecco's modified Eagle's/F-12 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml). BeWo b30-10 stable cells were grown in medium containing 0.5 mg/ml G418. Cells were grown to 90–100% confluency and transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen).

Transport Measurements

For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline (PBS)/CM (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.3) and [^{3}H] estrone sulfate (50nM). At the times indicated in the figure legends, the uptake was stopped by aspirating the uptake solution off and rapidly washing the cells with ice-cold PBS solution. Transport activity was also measured in cell monolayers cultured in transwell chambers (Costar, Cambridge, MA). To prepare cell monolayers, cells were seeded at a density of 1.0 x 10^5 cells per polycarbonate membrane (0.4μm pore size, collagen coated) in transwell cell chambers, which were placed in 12-well cluster plates. The volumes of medium inside and outside the chambers were 0.5 and 1.5 ml, respectively. Fresh medium was replaced

every day, and the cells were used between the 3rd and 4th days after seeding. To measure the cellular uptake of radiolabeled substrates, the reaction was initiated by adding substrate to the apical side or the basal side of the monolayers. After incubation for a specified period, the uptake medium was aspirated and discarded, and the membrane was rapidly washed three times with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquoted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well.

Cell surface biotinylation

Cell surface expression levels of hOAT4 and its mutants were examined using the membrane-impermeable biotinylation reagent, NHS-SS-biotin (Pierce). hOAT4 and its mutants were expressed in cells grown in 6-well plates using Lipofectamine 2000 as described above. After 24 h, the medium was removed and the cells were washed twice with 3 ml of ice-cold PBS/CM (pH 8.0). The plates were kept on ice, and all solutions were ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 μl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 with 1:100 protease inhibitor mixture (Sigma)). The cell lysates were cleared by

centrifugation at 16,000 x g at 4 °C. 50 µl of streptavidin-agarose beads (Pierce) was then added to the supernatant to isolate cell membrane protein. hOAT4 and its mutants were detected in the pool of surface proteins by SDS-PAGE and immunoblotting.

Electrophoresis and immunoblotting

Protein samples were resolved on 7.5% SDS-PAGE minigels and electroblotted on to polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS, 0.05% Tween 20, washed, and incubated for 1 h at room temperature with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected by SuperSignal West Dura extended duration substrate kit (Pierce). Nonsaturating, immunoreactive protein bands were quantitated by scanning densitometry with the FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA).

Data Analysis

Each experiment was repeated a minimum of three times. The statistical analysis given was from multiple experiments. Statistical analysis was performed using Student's paired t tests. A p value of = 0.05 was considered significant.

Results

Transport activity of hOAT4 and hOAT4-Δ

To investigate the interaction of hOAT4 with PDZ proteins, we constructed hOAT4-Δ, which lacks the last three amino acids (PDZ consensus binding site). Measurement of ^{3}H-labeled estrone sulfate transport into LLC-PK1 cells transfected with hOAT4 and hOAT4-Δ showed that both transporter proteins induced significant uptake as compared to that of mock control (Fig 1a). To facilitate the detection of these transporter proteins, epitope FLAG was tagged to the amino terminus of hOAT4 and hOAT4-Δ so that the epitope would not be expected to interfere with interaction of the carboxyl terminus with PDZ proteins. As shown in Fig 1b, the pattern of the transport activity FLAG-tagged hOAT4 and hOAT4-Δ mimicked their untagged counter parts, suggesting that the tagged transporters retained the functional properties of their parental transporters.

Interaction of FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ with PDZK1 and NHERF1

To facilitate the further characterization of hOAT4 and hOAT4-Δ with PDZ proteins, we established LLC-PK1 cells stably expressing of both transporters. Transfection of PDZ proteins PDZK1 and NHERF1 into LLC-PK1 cells (plated on 12-well plates) stably expressing FLAG-tagged hOAT4 significantly increased transport activity of the transporter (Fig. 2a). Such increase in transport activity was accompanied by an increase in cell surface expression of the transporter (Fig. 2b) without a change in its total cell expression (Fig. 2c). In contrast, transfection of PDZ proteins PDZK1 and NHERF1 into LLC-PK1 cells stably expressing

FLAG-tagged hOAT4-Δ had no effect on transport activity of the transporter (Fig. 3).

Interaction of FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ with PDZK1 and NHERF1 in polarized LLC-PK1 cells

The above experiments were carried out in LLC-PK1 cells plated in 12-well plates. When plated on transwells, LLC-PK1 cells polarized into apical and basolateral membrane domains, mimicking kidney proximal tubule cells in vivo. PDZ proteins have been shown to modulate the function of transporters at both the apical and the basolateral membranes (12,13). Estrone sulfate uptake was measured at both the apical and the basolateral membranes. Both the apical and basolateral membranes induced significant amount of uptake in cells expressing FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ as compared to that of mock cells (not shown). The estrone sulfate uptake was then measured in polarized cells after the transfection of PDZK1 or NHERF1. As shown in Fig. 4, both PDZK1 and NHERF1 induced an increased transport activity of hOAT4 at the apical side, whereas there was no effect of PDZK1 and NHERF1 on hOAT4 activity on the basolateral side. In cells expressing hOAT4-Δ, PDZK1 and NHERF1 had no effect on transport activity at both the apical and the basolateral sides (not shown).

Effect of PDZK1 and NHERF1 on hOAT4 in BeWo cells

In addition to being expressed in the kidney, hOAT4, PDZK1 and NHERF1 are also abundantly expressed in human placenta (11). To explore whether PDZK1 and NHERF1 has any effect on hOAT4 in placenta, we transfected PDZK1 and NHERF1 into human placenta BeWo cells stably expressing hOAT4 followed by

measurement of estrone sulfate uptake into either the apical or the basolateral sides of the cells. As shown in Fig. 5, transfection of PDZK1 and NHERF1 had no effect on hOAT4 function on both the apical and the basolateral sides.

Discussion

hOAT4 belongs to a family of organic anion transporters, which play critical roles in the body disposition of clinically important drugs. hOAT4 is abundantly expressed in the kidney and placenta. Although the regulation of hOAT4 in the kidney has begun to be explored, its regulation in the placenta is largely unknown. We have recently shown that hOAT4 activity in placental BeWo cells was down regulated by both activation of protein kinase C and pregnancy-specific hormones progesterone. However, progesterone regulates hOAT4 activity by mechanisms independent of PKC pathway (14). In the current study, we compared the interaction of hOAT4 with two PDZ proteins PDZK1 and NHERF1 in kidney LLC-PK1 cells and human placental BeWo cells.

Miyazaki et al. previously showed (11) that PDZK1 and NHERF1 increased hOAT4 activity in HEK293 cells, which was accompanied by an increased cell surface expression of the transporters. However, whether such increase in cell surface expression was caused by an increase in total protein synthesis or was caused by a trafficking of already existent intracellular hOAT4 to the cell surface was not examined. Furthermore, PDZ proteins have been shown to modulate transporter activity at both the apical and the basolateral surface of the transport epithelia. For example, it has been shown that PDZK1 regulated transport activity of a urate/anion exchanger URAT1 on the apical side of the kidney proximal tubule cells (12) and an organic anion transporting protein Oatp1a1 at the basolateral side of liver hepatocytes (13). HEK293 cells are nonpolarized cells and therefore, polarized regulation of hOAT4 by PDZ proteins could not be explored. In the

current study, we expanded their previous observation by showing that PDZK1 and NHERF1 only enhanced transport activity of hOAT4 at the apical side but not the basolateral side, and that the increased transport activity was due to an increased cell surface expression without affect the total cell expression of the transporter. Our finding suggests that PDZ proteins affect hOAT4 activity by affecting its trafficking between cell surface and intracellular compartment. PDZK1 and NHERF1 also increased hOAT4 activity in COS-7 cells, another kidney cell line (data not shown). Therefore, modulation of hOAT4 activity by PDZK1 and NHERF1 is probably the feature of kidney cells.

In the current study we also provided interesting data showing that although hOAT4, PDZK1 and NHERF1 are all present in the placenta (11), transfection of PDZK1 and NHERF1 into placental BeWo cells expressing hOAT4 had no effect on the transport activity of the transporter on both the apical and the basolateral sides, which is in contrast to what we observed of the interactions between these proteins in kidney cells LLC-PK1 and COS-7. This finding suggests that interaction of hOAT4 with PDZ proteins may depend on specific organ. It is possible that in placenta, a different set of proteins regulate OAT4 function. However, we can't exclude the possibility that hOAT4 in BeWo cells was already fully engaged in the interaction with endogenously expressed PDZK1, NHERF1 or similar PDZ proteins. Further increase in PDZ protein level will not increase hOAT4 activity.

In conclusion, we showed that PDZ proteins PDZK1 and NHERF1 are regulators of hOAT4 function in kidney cells, whereas the same set of PDZ

proteins has no effect on hOAT4 function in placenta cells, suggesting that the interacting partners of hOAT4 in placenta maybe different from that in kidney.

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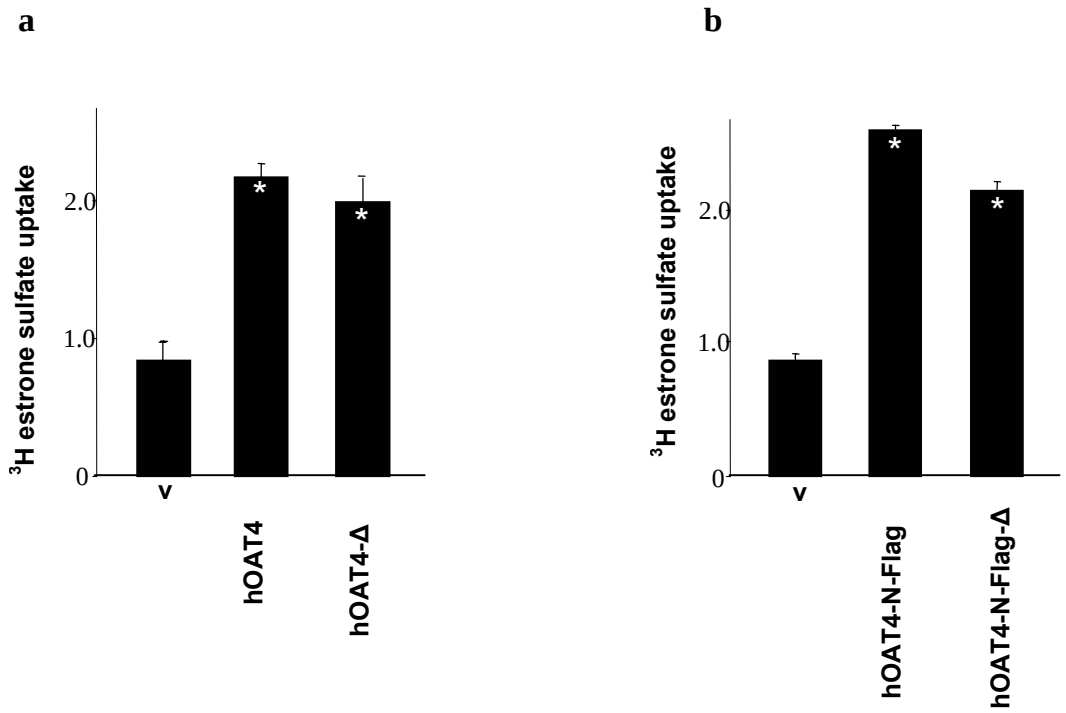

Fig 1. Estrone sulfate uptake.

a. Estrone sulfate uptake into cells expressing hOAT4 and hOAT4-Δ. [^{3}H] estrone sulfate uptake (4 min, 100 nM) was measured. Uptake activity was expressed as a percentage of the uptake measured in mock cells (v). The results represent data from three experiments. Values are mean±S.E. (*n* = 3). *Asterisks* indicate values significantly different ($p < 0.05$) from that of mock control. b. Estrone sulfate uptake into cells expressing FLAG-tagged hOAT4 and hOAT4-Δ. Uptake activity was expressed as a percentage of the uptake measured in mock cells (v). The results represent data from three experiments. Values are mean±S.E.(*n* = 3). *Asterisks* indicate values significantly different ($p < 0.05$) from that of mock control.

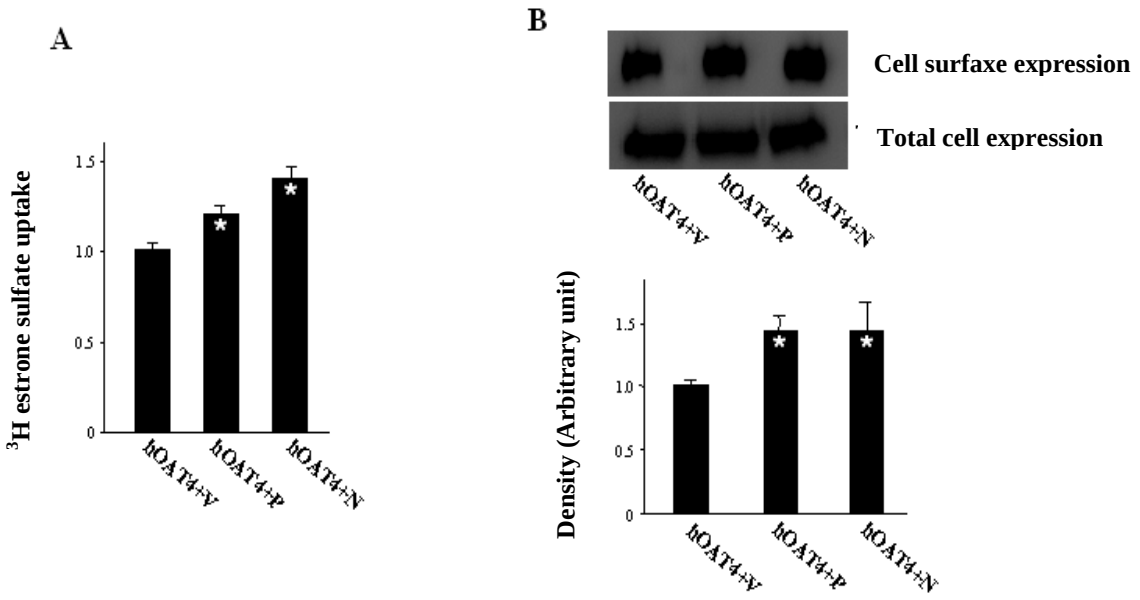

Fig 2. Effect of PDZK1 and NHERF1 on FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ.

a. Functional effect of PDZK1 and NHERF1 on FLAG-tagged hOAT4 in LLC-PK1 cells. FLAG-tagged hOAT4 expressing cells were transiently transfected with PDZK1, NHERF1 or vector followed by [^{3}H]estrone sulfate uptake (4 min, 100 nM). V: vector; P: PDZK1; N: NHERF1. Uptake activity was expressed as a percentage of the uptake measured in cells expressing FLAG-tagged hOAT4 alone. The results represent data from three experiments. Values are mean±S.E. (*n* = 3).

b. Effect of PDZK1 and NHERF1 on cell surface and total cell expression of FLAG-tagged hOAT4. *Top panel:* For cell surface expression cells stably expressing FLAG-tagged hOAT4 were biotinylated and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by Western blotting with anti-FLAG antibody (1:500). V: vector; P: PDZK1; N: NHERF1. For total cell expression, cells stably expressing FLAG-tagged hOAT4 were lysed and separated by SDS-PAGE, followed by Western blotting with anti-FLAG antibody (1:500). V: vector; P: PDZK1; N: NHERF1. *Bottom panel:* Densitometry analysis of the ratio of the band densities of cell surface expression vs. total cell expression. *Asterisks* indicate values significantly different ($p < 0.05$) from that of control (hOAT4 +V).

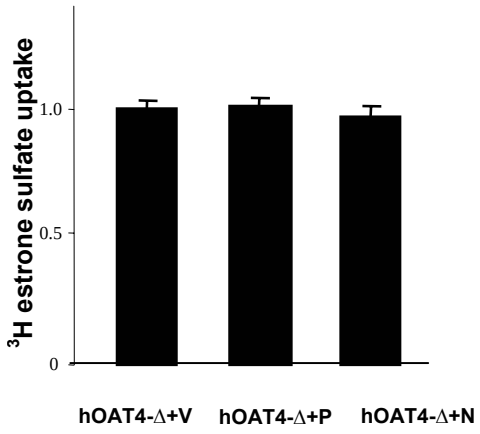

Fig 3. Functional effect of PDZK1 and NHERF1 on FLAG-tagged hOAT4-Δ in LLC-PK1 cells.

hOAT4-Δ expressing cells were transiently transfected with PDZK1, NHERF1 or vector (v) followed by [^{3}H]estrone sulfate uptake (4 min, 100 nM). Uptake activity was expressed as a percentage of the uptake measured in cells expressing FLAG-tagged hOAT4-Δ alone. The results represent data from three experiments. Values are mean±S.E.(*n* = 3).

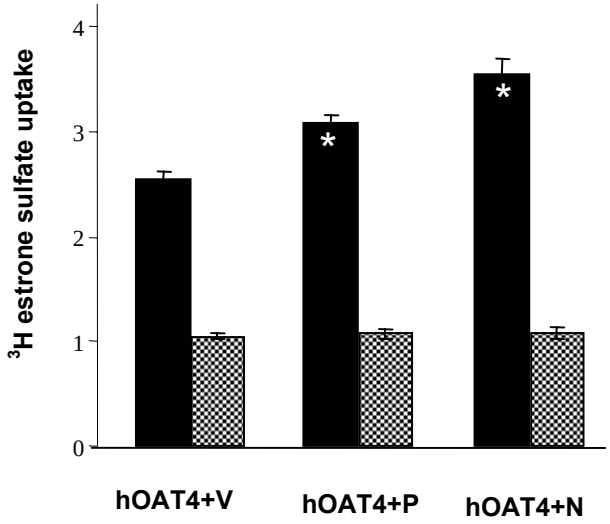

Fig 4. Interaction of FLAG-tagged hOAT4 and FLAG-tagged hOAT4-Δ with PDZK1 and NHERF1 in polarized LLC-PK1 cells.

Cells expressing FLAG-tagged hOAT4 were seeded on transwells followed by transient transfection of PDZK1 or NHERF1. Directional uptake of estrone sulfate (100 nM, 4 min) from either apical sides (*solid columns*) or basal sides (*dotted columns*) were then performed. V: vector; P: PDZK1; N: NHERF1.Uptake activity was expressed as a percentage of the basal uptake measured in cells expressing FLAG-tagged hOAT4 alone. The results represent data from three experiments. Values are mean ± S.E. (*n* = 3).

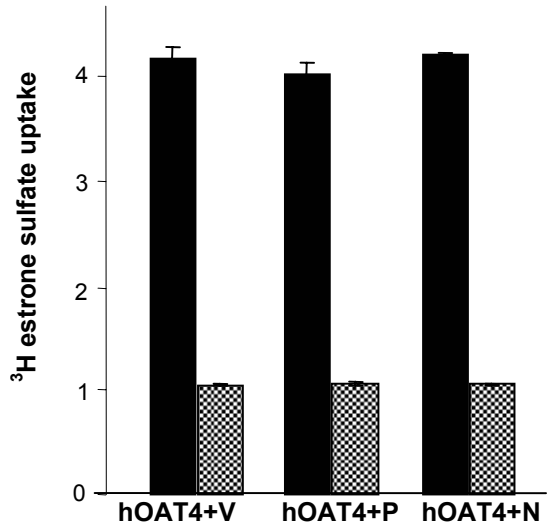

Fig 5. Interaction of hOAT4 with PDZK1 and NHERF1 in polarized BeWo cells.

Cells expressing hOAT4 were seeded on transwells followed by transient transfection of PDZK1 or NHERF1. Directional uptake of estrone sulfate (100 nM, 4 min) from either apical sides (*solid columns*) or basal sides (*dotted columns*) were then performed. V: vector; P: PDZK1; N: NHERF1. Uptake activity was expressed as a percentage of the basal uptake measured in cells expressing hOAT4 alone. The results represent data from three experiments. Values are mean ± S.E. (*n* = 3).

Chapter 6:

Functional characterization of a human organic anion transporter hOAT4 in placental BeWo cells

This chapter was written in the style of and published in European journal of pharmaceutical sciences (Zhou F, Illsley NP, You G. Eur J Pharm Sci. 2006 Apr; 27(5):518-23.)

Abstract

Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters which play critical roles in the body disposition of clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories. hOAT4 is expressed in the placenta and kidney. In the current study, we stably transfected hOAT4 into human placental BeWo cells and the functional properties of hOAT4 and its regulation were investigated in these cells. hOAT4-mediated uptake of estrone sulfate, a protypical organic anion for hOAT4, was dose- and time-dependent, and saturable (K_m = 4.2 μM). The substrate specificity of hOAT4 includes various steroid sulfates, such as β-estradiol-3,17-disulfate, 17-β-estradiol-3-sulfate, β-estradiol-3-sulfate, and dehydroepiandrosterone-3-sulfate (DHEAS), but does not include *p*-aminohippuric acid (PAH) and tetraethylammonium (TEA). Pre-incubation of hOAT4-expressing BeWo cells with phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), both of which are protein kinase C (PKC) activators, acutely inhibited the transport activity. The inhibition by PDBu resulted in a decreased V_{max} without significant affecting the K_m. Establishment of hOAT4-expressing BeWo cells provided useful tool for further pharmacological and molecular biological studies of placental transport of organic anions mediated by this carrier.

Keywords: Organic anion transport; Transporter; Placenta; BeWo cells

1. Introduction

Organic anion transporters (OATs) play essential roles in the body disposition of clinically important anionic drugs including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories (You, 2002, You, 2004a and You, 2004b). Several OAT isoforms have been cloned by different laboratories (Cha et al., 2000, Cihlar et al., 1999, Kusuhara et al., 1999, Lopez-Nieto et al., 1997, Lu et al., 1999, Sekine et al., 1998, Sekine et al., 1997, Sweet et al., 1997, Wolff et al., 1997 and Youngblood and Sweet, 2004). These OATs have distinct cellular and tissue distributions. OAT1 and OAT3 are predominantly expressed in the kidney and the brain. OAT2 is predominantly expressed in the liver. OAT4 is present mainly in the placenta and the kidney. OAT5 is expressed in the kidney.

In primates, plasma concentrations of unconjugated estrone, estradiol, and estriol increase linearly with advancing gestation. After approximately week 9 of human pregnancy, the placenta becomes the main source of maternal estrogens. It is believed that estrogen biosynthesis in the placenta uses dehydroepiandrosterone-3-sulfate (DHEAS), a precursor produced in large amount by the fetal adrenals. When fetal adrenals are stimulated by adrenocorticotropic hormone in the second trimester, synthesis of DHEAS increases. Accumulation of excess DHEAS is associated with intrauterine growth retardation (IUGR) (Rabe et al., 1983), because of inhibition of cell proliferation and differentiation by DHEAS (Herrington et al., 1990, Mohan and Cleary, 1989 and Schulz et al., 1992). IUGR can be characterized as a condition where the

fetus has failed to achieve its genotypical growth potential. IUGR is associated with increased prenatal morbidity, higher incidence of neurodevelopment impairment, and increased risk for a number of diseases in adulthood, such as cardiovascular disease and diabetes (Blair and Stanley, 1990 and Hales and Barker, 1992). Therefore, efficient uptake of DHEAS by the placenta is required not only for the production of estrogens but also for the protection of fetus from the cytotoxicity of DHEAS. Human organic anion transporter 4 (hOAT4) plays a major role in the placental uptake of fetal-derived DHEAS and other steroid sulfates (Ugele et al., 2003). In addition to its physiological role, hOAT4 is also a multi-specific xenobiotics transporter interacting with numerous environmental toxins and clinically important drugs (You, 2002, You, 2004a and You, 2004b). As a result, unwanted exposure of the fetus to xenobiotics may occur through hOAT4-mediated pathway. Despite the vital importance of hOAT4 in fetal development, how the transporter is regulated in placenta is completely unknown. As a first step to address this issue, we established human placental cells BeWo, stably expressing hOAT4 and explored its regulation by protein kinase C.

2. Materials and methods

[^{3}H] estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical (Rockford, IL). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO). BeWo b30-10 cell line was provided by Dr. Nicholas P. Illsley (Robert Wood Johnson Medical School, University of Medicine & Dentistry of New Jersey, Piscataway, NJ).

2.1. Generation of cells stably expressing hOAT4

Parental BeWo b30-10 cells were grown in Dulbecco's modified Eagle's/F-12 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO_2 atmosphere at 37 °C. Cells were seeded at 5×10^5 per well of 6-well cluster plate 24 h before transfection. For transfection of hOAT4 cDNA plasmid, a LipofectAMINE 2000 reagent was used following the manufacturer's instruction. After 7–8 days of selection in medium containing 0.5 mg/ml geneticin (G418; Invitrogen, Carlsbad, CA), resistant colonies were replated to 96 wells for cloning, expansion, and analyzing positive clones.

2.2. Transport measurement

Cells were treated with each reagent at 37 °C for certain time periods as shown in the figure legends. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/Ca^{2+}/Mg^{2+} (PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mM $CaCl_2$, and

1 mM $MgCl_2$, pH 7.4) and [^{3}H]estrone sulfate. At the times indicated in the figure legends, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2N NaOH, neutralized in 0.2N HCl, and aliquoted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values were mean ± S.E. (*n* = 3).

2.3. Cell-surface biotinylation

Cell-surface expression levels of hOAT4 were examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin (Pierce Chemical). The BeWo b30-10 cells stably expressing hOAT4 and parental BeWo b30-10 cells were seeded onto 6-well plate at 8×10^5 per well. After 48 h, the medium was removed, and the cells were washed twice with 3 ml of ice-cold PBS, pH 8.0. The plates were kept on ice, and all solutions were kept ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS) in two successive 20-min incubations on ice with very gentle shaking. The reagent was freshly prepared for incubation. After biotinylation, each well was briefly rinsed with 3 ml of PBS containing 100 mM glycine, and then incubated with the same solution for 20 min on ice to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 µl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and protease inhibitors phenylmethylsulfonyl fluoride, 200 µg/ml, and leupeptin, 3 µg/ml, pH 7.4). The unlysed cells were removed by centrifugation at 13,000 rpm at 4 °C. Streptavidin-agarose beads (50 µl; Pierce Chemical) were

then added to the supernatant to isolate cell membrane protein. hOAT4 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-hOAT4 antibody (Alpha Diagnostic International, Inc., San Antonio, TX).

2.4. Electrophoresis and Western blot

Protein samples (with equal amount) were resolved on 7.5% SDS-PAGE minigels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS/0.05% Tween, washed, and incubated overnight at 4 °C with polyclonal anti-hOAT4 antibody (1:500, Alpha Diagnostic International, Inc.). The membranes were washed and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000), and signals were detected by SuperSignal West Dura Extended Duration Substrate Kit (Pierce Chemical).

3. Results

3.1. Generation of BeWo cells stably expressing hOAT4

To make stable hOAT4-expressing clones in BeWo cells, we cloned the cDNAs, encoding hOAT4 behind the cytomegalovirus promoter in the mammalian expression vector pcDNA3.1 (−), which contains the *neo* gene for selection with G418. After 2 weeks of selection, seven clones were obtained from cell populations transfected with the control plasmid (vector alone), and six clones were obtained from populations transfected with pcDNA3.1 (−)–hOAT4 plasmid. Preliminary studies measured [^{3}H] estrone uptake in clones of hOAT4-expressing cells (data not shown). Clone exhibiting high level of uptake was chosen for further studies.

The expression of hOAT4 protein in BeWo cells was first examined by immunoblot analysis of total cell proteins (Fig. 1a). In total cell lysate, anti-hOAT4 antibody recognized a product with a molecular mass of ~ 83 kDa in hOAT4-transfected cells (Fig. 1a, lane 1), which corresponded to the fully glycosylated form of hOAT4 (Zhou et al., 2005). Such protein was not detected in pcDNA vector-transfected cells (Fig. 1a, lane 2). The expression of hOAT4 protein in BeWo cells was then examined by immunoblot analysis of cell surface proteins (Fig. 1b). Cell-surface proteins were biotinylated with the membrane-impermeant reagent NHS-SS-biotin, isolated with streptavidin-coated beads, and immunoblotted with anti-hOAT4 antibody. Again, anti-hOAT4 antibody recognized a product with a molecular mass of ~83 kDa in hOAT4-transfected cells (Fig. 1b,

lane 1) but not in pcDNA vector-transfected cells (Fig. 1b, lane 2). Therefore, hOAT4 in BeWo cells contained fully processed carbohydrates.

3.2. Functional characterization of hOAT4

Because this is the first study using BeWo cells as an expression system for hOAT4, the functional properties of hOAT4 in these cells were characterized. The time course for [^{3}H] estrone sulfate uptake was compared in vector-transfected and hOAT4-transfected cells. As expected, uptake was markedly faster into hOAT4-expressing cells. In hOAT4-expressing cells, uptake increased linearly for approximately 5 min and reached a steady state between 10 and 20 min (data not shown). Therefore, an uptake period of 2 min (initial rate) was chosen for future studies.

The kinetics of estrone sulfate transport was first studied using 2-min uptake points. The initial rate of estrone sulfate uptake over a wide range of estrone sulfate concentrations was determined (Fig. 2). The transport of estrone sulfate across the cell membrane was saturable (Fig. 2a). Based on Eadie–Hofstee plot analysis (Fig. 2b), the K_m value for estrone sulfate was 4.2 ± 0.6 μM and V_{max} was 0.025 ± 0.001 pmol/(mg 2 min).

3.3. Substrate specificity

We examined the substrate specificity of the carrier in competition experiments (Fig. 3). *cis*-Inhibitory effects of [^{3}H] estrone sulfate were observed for unlabeled steroid sulfates, including estrone-3-sulfate, β-estradiol-3,17-disulfate, 17β-estradiol-3-sulfate, β-estradiol-3-sulfate, and

DHEAS. The transport was unaffected by *p*-aminohippurate (PAH) and organic cation tetraethylammonium.

3.4. Effect of activation of PKC on estrone sulfate transport

hOAT4 sequence contains multiple sites for protein kinase C. Therefore, the potential involvement of this kinase in hOAT4 function was examined. We treated the hOAT4-expressing BeWo cells with the PKC activator, phorbol 12-myristate 13-acetate (PMA). Our results showed that, when cell monolayers were treated with 1 μM PMA for 15 min, the hOAT4-mediated estrone sulfate transport was decreased by 30% as compared to that of untreated monolayers (Fig. 4a). This PMA- induced inhibition was time- and concentration-dependent (data not shown). The effect of various other PKC activators on estrone sulfate transport was also studied. Like PMA, phorbol 12,13-dibutyrate (PDBu) (the active phorbol ester) inhibited the estrone sulfate uptake. In contrast, 4α-PDD (the inactive phorbol ester) did not affect estrone sulfate uptake. Staurosporine, a potent inhibitor of PKC, blocked the inhibitory effect by PMA (Fig. 4b).

To further examine the mechanism of PKC-induced down-regulation of estrone sulfate transport, we determined [^{3}H] estrone sulfate uptake at different substrate concentrations. An Eadie–Hofstee analysis of the derived data (Fig. 5) showed that pre-treatment with PDBu resulted in a decreased V_{max} (0.025 ± 0.001 pmol/(μg 2 min) with untreated cells, and 0.006 ± 0.001 pmol/(μg 2 min) in the presence of PDBu) with no significant change in the affinity for estrone sulfate (4.2 ± 0.6 μM with untreated cells and

3.0 ± 0.5 μM in the presence of PDBu). Determination of the protein concentrations in control wells confirmed that PDBu treatment did not change the total protein content of the cultures (data not shown).

4. Discussion

In human placenta, hOAT4 plays an important role in the uptake of fetal-derived dehydroepiandrosterone-3-sulfate (DHEAS) and other steroid sulfates (Ugele et al., 2003) for subsequent synthesis of estrogens critical for the maintenance of pregnancy. Accumulation of excess DHEAS is associated with intrauterine growth retardation (Rabe et al., 1983). Therefore, efficient uptake of fetal-derived DHEAS by hOAT4 is required not only for the production of estrogens but also for the protection of fetus from the cytotoxicity of DHEAS. Apart from its physiological role, hOAT4 is also a multi-specific xenobiotics transporter interacting with numerous environmental toxins and clinically important drugs (You, 2002, You, 2004a and You, 2004b). As a result, unwanted exposure of the fetus to xenobiotics may occur through hOAT4-mediated pathway. Despite the significant role of hOAT4 in fetal development, there has been no report concerning the regulation of this transporter. To begin to understand the regulation of this transporter, we established human placental cells BeWo, stably expressing hOAT4 and explored its regulation by PKC.

BeWo cells offer several useful advantages for study of the cloned organic anion transporter. (1) They have many characteristics of human placenta and have been very useful in understanding other placental epithelial transport processes and cellular functions (Emoto et al., 2002, Manley et al., 2005 and Vardhana and Illsley, 2002). (2) This cell line does not express endogenous OATs. Therefore, expression of hOAT4 in BeWo cells will allow us to dissect the transport characteristics of hOAT4 in a relevant mammalian system without the possibly

confounding effects of other organic anion transporters. (3) They possess endogenous protein kinase signaling pathways and provide a good experimental model system for studying the regulatory mechanisms of many transport processes (Huang and Swaan, 2001 and Sakai et al., 1997).

In this study, hOAT4-transfected BeWo cells were shown to be a valid model system for the functional analyses. The affinity (K_m) of hOAT4 for the protosubstrate estrone sulfate in BeWo cells (4.2 μM) and its inhibition profile were comparable with those obtained from other systems (Cha et al., 2000).

PMA and PDBu, two specific PKC activators, induced a time- and dose-dependent inhibition of uptake of estrone sulfate mediated by hOAT4 in BeWo cells. Furthermore, staurosporine, a PKC inhibitor, reversed the PMA-induced decrease of uptake of estrone sulfate. These results suggest that inhibition of uptake of estrone sulfate by PMA and PDBu is due to the activation of PKC.

Kinetic analysis revealed that PDBu treatment resulted in a decrease in the V_{max} but not the K_m of the uptake of estrone sulfate mediated by hOAT4 in BeWo cells. PKC-induced direct phosphorylation has been reported for other transporters (Li et al., 1998, Ramamoorthy et al., 1998 and Vaughan et al., 1997). In a previous study on mouse organic anion transporter mOAT1 (You et al., 2000), we showed that PKC down-regulated mOAT1 function, kinetically revealed as a decrease in V_{max}. However, such decrease in V_{max} was not due to PKC-induced phosphorylation. Further study by Wolff et al. (2003) showed PKC-induced

down-regulation of human organic anion transporter hOAT1 function occurred through carrier internalization. Whether hOAT4 shares the same regulatory pathway as other OATs (Takeda et al., 2000, Wolff et al., 2003 and You et al., 2000) is an important issue, which needs further investigation.

In conclusion, our present study suggests that hOAT4-transfected BeWo cells can serve as an in vitro model system for studying placental pharmacology and biology of the cloned hOAT4. Our study also presents data characterizing the impact of PKC on the function of the cloned hOAT4. The results contribute to our understanding of the regulatory mechanisms of the organic anion transporters.

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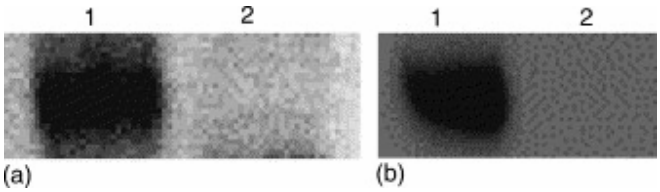

Fig 1. Characterization of BeWo cells stably expressing hOAT4.

(a) Total cell expression of hOAT4. Cells expressing hOAT4 and pcDNA vector-transfected cells were lysed, and their proteins were separated by SDS-PAGE and visualized by immunoblot analysis using anti-hOAT4 antibody.

(b) Cell surface expression of hOAT4. Cells expressing hOAT4 and pcDNA vector-transfected cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated, and visualized as above.

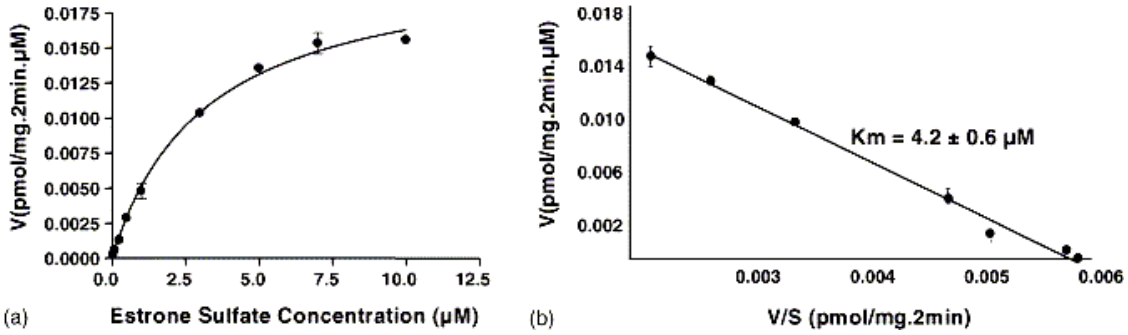

Fig 2. Kinetic analysis of hOAT4-mediated estrone sulfate transport.

(a) Kinetic characteristics were determined at substrate concentration ranging from 0.05 to 10 μM (2-min uptake). The data represent uptake into pcDNA–hOAT4 transfected cells minus uptake into pcDNA vector-transfected cells. Values are mean ± S.E. (*n* = 3).

(b) Transport kinetic values were calculated using the Eadie–Hofstee transformation.

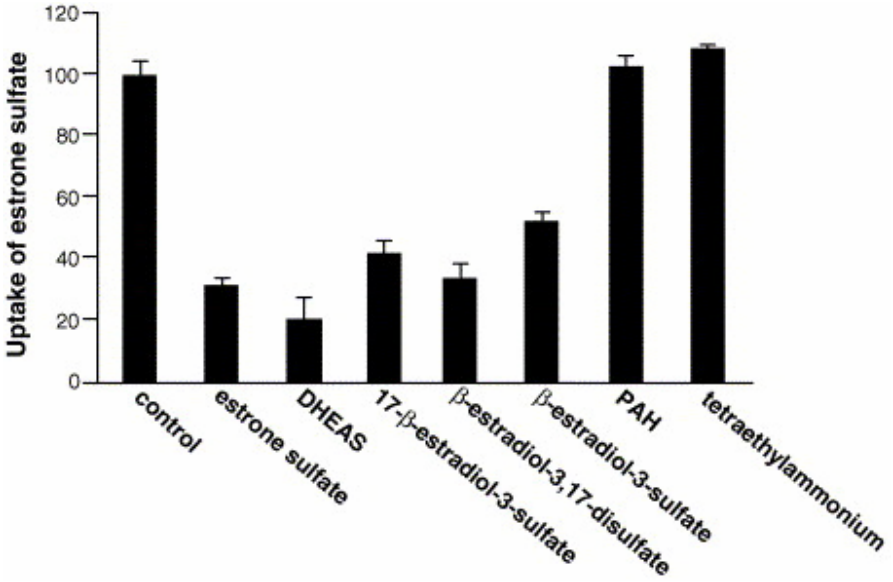

Fig 3. Substrate specificity of hOAT4.

The substrate selectivity was obtained in competition experiments. Inhibition of [^{3}H] estrone sulfate (100 nM) by various organic anions (5 μM) was determined. The data are presented as percent of control uptake.

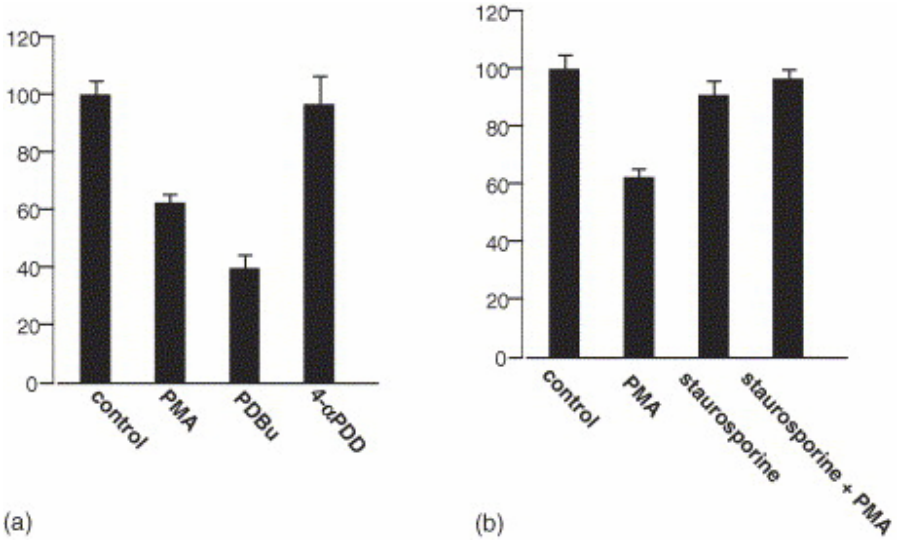

Fig 4. Specificity of PKC activation on estrone sulfate transport.

(a) Cells were pre-treated with or without PMA, PDBu, or 4-αPDD for 15 min and uptake of [^{3}H] estrone sulfate was measured. The data are presented as percent of control uptake.

(b) Reversal of PMA effect by staurosporine. Cells were treated with or without PMA (1 μM) in the presence and absence of staurosporine (2 μM) for 15 min followed by measuring the uptake of [^{3}H] estrone sulfate. The data are presented as percent of control uptake.

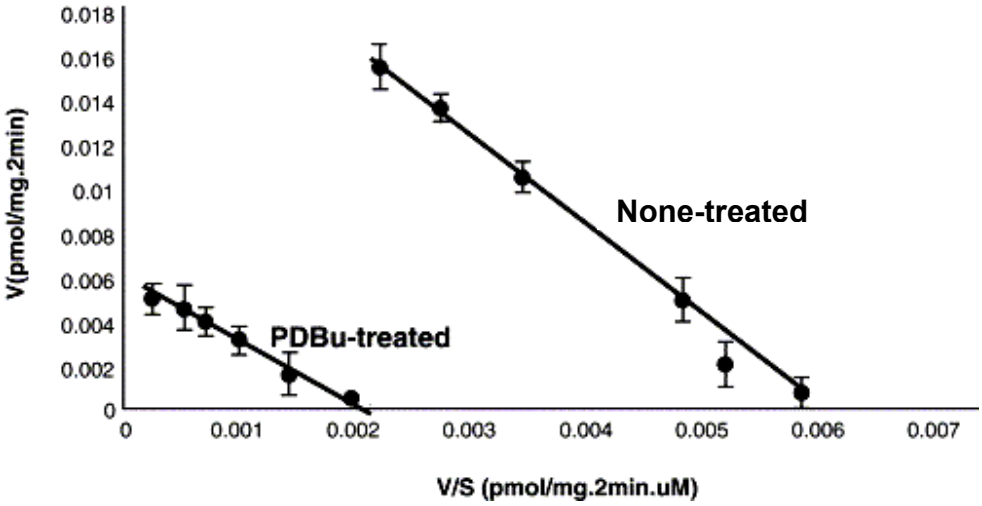

Fig 5. Effect of PDBu on the kinetics of estrone sulfate transport.

Cells expressing hOAT4 and pcDNA vector were pre-treated with or without PDBu 1 μM for 15 min and initial uptake of [^{3}H] estrone sulfate was measured at 0.05–10 μM estrone sulfate. The data represent uptake into hOAT4-transfected cells minus uptake into pcDNA vector-transfected cells. Values are mean ± S.E. (*n* = 3).

Chapter 7:

Characterization of an organic anion transport system in a placental cell line

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Abstract

Transporters within the placenta play a crucial role in the distribution of nutrients and xenobiotics across the maternal-fetal interface. An organic anion transport system was identified on the apical membrane of the rat placenta cell line HRP-1, a model for the placenta barrier. The apical uptake of ^{3}H-labeled organic anion estrone sulfate in HRP-1 cells was saturable (K_m = 4.67 μM), temperature and Na^+ dependent, Li^+ tolerant, and pH sensitive. The substrate specificity of the transport system includes various steroid sulfates, such as β-estradiol 3,17-disulfate, 17β-estradiol 3-sulfate, and dehydroepiandrosterone 3-sulfate (DHEAS) but does not include taurocholate, *p*-aminohippuric acid (PAH), and tetraethylammonium. Preincubation of HRP-1 cells with 8-bromo-cAMP (a cAMP analog) and forskolin (an adenylyl cyclase activator) acutely stimulated the apical transport activity. This stimulation was further enhanced in the presence of IBMX (a phosphodiesterase inhibitor). Together these data show that the apical membrane of HRP-1 cells expresses an organic anion transport system that is regulated by cellular cAMP levels. This transport system appears to be different from the known taurocholate-transporting organic anion-transporting polypeptides and PAH-transporting organic anion transporters, both of which also mediate the transport of estrone sulfate and DHEAS.

Keywords: placenta; organic anion; transporters

Introduction

The placenta forms the sole structural barrier between the mother and the developing fetus and performs many functions that are essential for normal fetal development. One of the major functions of the placenta is to mediate the transfer of nutrients from the mother to the fetus and to eliminate metabolic waste products from the fetus (2, 9, 16). This function is facilitated by the polarized expression of various transporters in the maternal-facing brush border membrane and the fetal-facing basal membrane of the placenta epithelium. The specificity of these transporters is, however, not limited to their physiological substrates. Xenobiotics bearing structural similarity to the physiological substrates have the potential to be recognized by these transporters. These compounds include therapeutic agents and environmental toxins. Therefore, the transporters expressed in the placenta crucially influence the distribution of these xenobiotics across the maternal-fetal interface (2, 9, 16).

Many xenobiotics are organic anions or are metabolized to organic anions. Their absorption and/or elimination into and/or from the body are handled by several groups of organic anion transporters (OATs) (11, 34). Although the roles of these transporters in other tissues such as kidney and liver have been extensively studied, their roles in placenta have just begun to be explored. The placenta is known to transport various organic anions such as steroid sulfates, bile acids, and thyroid hormones (1, 24, 29). Recently, several OATs have been identified in the placenta. At gene level, the mRNAs for organic anion transporting polypeptides (OATPs), Oatp1, Oatp2, Oatp3, Oatp4, Oatp5, Oatp9 and Oatp12, have been

detected, with various degrees of abundance, in rat placenta (19). However, whether any of these genes produces a functional transporter in this organ is not known. At the protein level, OAT4 and OATP-B have been immunohistochemically localized to the basolateral membranes of human placenta and human placenta-derived BeWo cells (30, 31). This basolateral localization is consistent with the functional expression of a transport system in basal syncytiotrophoblast membrane vesicles. OATP-E protein has been localized to the apical membrane of human placenta by the immunohistochemical approach (26). However, no functional data correlating with this apical expression are yet available. Furthermore, no information is available for the placental regulation of organic anion transport. In the present study, we identified, by functional analyses, an organic anion transport system on the apical membrane of the placenta cell line HRP-1. We also dissected the transport mechanism and investigated its intracellular regulatory pathways.

The HRP-1 cell line is derived from normal rat placenta and appears morphologically similar to, and retains characteristic expression of, cellular markers of labyrinthine trophoblast cells, in which the bulk of maternal-fetal nutrient/waste transfer occurs (13, 14, 20). This cell line has proved to be a useful in vitro model system to study function and regulation of several transporters, including glutamate transporters (22), fatty acid transporters (17), and a facilitative glucose transporter (5). Therefore, we have chosen this cell line for the present studies.

Materials and Methods

Materials.

[^{3}H] estrone sulfate, [^{3}H] dehydroepiandrosterone 3-sulfate (DHEAS), and [^{14}C] taurocholate were purchased from Perkin Elmer Life Science. Culture media were obtained from Invitrogen Life Technologies. All other chemicals were from Sigma.

Cell culture.

HRP-1 cells were maintained in RPMI 1640 culture medium, pH 7.4, containing 1 mM sodium pyruvate, 50 μM 3-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were seeded in 75-cm^2 flasks and incubated at 37°C with 5% CO_2.

Transport measurements.

Transport was measured in HRP-1 cell monolayers cultured in transwell chambers (Costar, Cambridge, MA). To prepare cell monolayers, cells were seeded at a density of 1.2 x 10^5 cells per polycarbonate membrane (0.4-μm pore size, collagen coated) in transwell cell chambers, which were placed in 12-well cluster plates. The volumes of medium inside and outside the chambers were 0.5 and 1.5 ml, respectively. Fresh medium was replaced every 2 days, and the cells were used between the 3rd and 4th days after seeding. Transport was measured in Dulbecco's phosphate-buffered saline (PBS), containing 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ supplemented with 5 mM D-glucose. To measure the cellular uptake of

radiolabeled substrates, the reaction was initiated by adding each buffer containing substrate to the apical side of the monolayers. After incubation for a specified period, the uptake medium was aspirated and discarded, and the membrane was rapidly washed three times with ice-cold PBS. The cell monolayers on the membrane were solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquoted for liquid scintillation counting. The protein concentration was determined using the Bradford dye-binding procedure (3). For the inhibition studies, uptake of radiolabeled substrates was measured in the presence of unlabeled compounds as indicated.

Data analysis and statistics.

Kinetic uptake parameters such as the concentration at half-maximal transport velocity (K_m, Michaelis-Menten constant), maximum uptake velocity (V_{max}), and passive membrane permeability coefficients (P_{diff}) were calculated using SigmaPlot software (SPSS, Chicago, IL) by nonlinear least squares regression analysis. Calculations utilized the following equation, where V is the total flux and [S] is the estrone sulfate concentration.

$$V = \frac{V_{max}[S]}{K_m + [S]} + P_{diff}[S]$$

Statistics.

To test the significance of differences between data sets, Student's *t*-test was performed.

Results

Kinetics of [^{3}H] estrone sulfate transport.

At confluence, HRP-1 cells form an epithelium with separate apical and basolateral membrane domains containing different complements of membrane proteins. Initial experiments were performed by measuring the apical uptake into HRP-1 cells of [^{3}H] estrone sulfate, a common substrate for members of the OATP family. As shown in Fig. 1, the apical uptake of [^{3}H] estrone sulfate increased linearly for ~5 min and reached a steady state between 5 and 10 min. Therefore, an uptake period of 3 min was chosen for future studies. The initial rate for the uptake of estrone sulfate over a wide range of estrone sulfate concentrations (50 nM-10 μM) was then determined (Fig. 2). Kinetic analysis revealed a saturable uptake mechanism, which was superimposed by a nonsaturable component, presumably simple diffusion. Fitting the data to *Eq. 1* yielded a K_m of 4.67 μM, a V_{max} of 1.13 pmol · mg^{-1} · min^{-1}, and a P_{diff} of 0.044 μl · mg^{-1} · min^{-1}.

Temperature dependence.

The uptake of estrone sulfate in HRP-1 cells was further characterized at a decreased temperature (Fig. 3). Incubation at 4°C significantly reduced the rate of uptake compared with that seen at room temperature, indicating that the transport of estrone sulfate is a temperature-dependent process.

Na^+ dependence.

The role of Na^+ in estrone sulfate uptake by HRP-1 cells was investigated in this study (Fig. 4). This was done by examining the effect of isosmotically replacing Na^+ (137 mM) in the incubation buffer with the monovalent cation choline

(137 mM choline chloride) or Li^+ (137 mM LiCl). The results showed that choline ion substitution for Na^+ led to a significant decrease in estrone sulfate uptake compared with that in cells incubated in Na^+ buffer. Replacement of Na^+ by Li^+ completely restored the stimulatory effect by Na^+, demonstrating that the transport function is Na^+ dependent and tolerates replacement of Na^+ by Li^+.

The role of Na^+ in estrone sulfate uptake was further examined using ouabain, a specific Na^+-K^+-ATPase inhibitor. Preincubation of HRP-1 cells with 1 mM ouabain significantly inhibited cell uptake of estrone sulfate (Fig. 5), suggesting that the transport is driven by the (transmembrane) Na^+ gradient established by Na^+-K^+-ATPase.

pH dependence.

In a separate experiment we examined the effect of varying the incubation buffer pH over the range of 5.0 to 8.5 (Fig. 6). The results showed a ~65–100% increase in estrone sulfate uptake at an acidic pH of 5.0 compared with uptake observed at pH 7.4 and pH 8.5. To test whether the uptake process is driven by an inwardly directed proton gradient, cells were pretreated separately with amiloride, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), and nigericin (Fig. 7). Amiloride is a specific inhibitor for Na^+/H^+ exchanger. FCCP, a protonophore, is an uncoupler of oxidative phosphorylation that equilibrates proton concentration across biological membranes. Nigericin is a $K+/H^+$ ionophore that mediate the electroneutral exchange of a proton for a potassium ion across biological membranes, leading to a decrease in membrane pH gradient without affecting the

membrane potential. None of these treatments blocked the increase of the uptake in the presence of an inwardly directed H^+ gradient (incubation medium pH 5.0), ruling out a H^+ cotransport process.

Substrate specificity.

We first examined the substrate selectivity of the transport system by competition studies (Fig. 8*A*). *Cis-Inhibitory* effects of [^{3}H] estrone sulfate were observed for unlabeled steroid sulfates, including estrone sulfate, ß-estradiol-3,17-disulfate, 17ß-estradiol-3-sulfate, and DHEAS. The transport was unaffected by *p*-aminohippurate (PAH), taurocholate, and tetraethylammonium.

We then further evaluated several radiolabeled compounds in terms of whether they were taken up into the cells via the same transport system. As shown in Fig. 8*B*, the uptake of [^{3}H] DHEAS was *cis*-inhibited by unlabeled DHEAS and estrone sulfate. Vice versa, the uptake of [^{3}H] estrone sulfate was *cis*-inhibited by unlabeled estrone sulfate and DHEAS (Fig. 8*A*). This mutual inhibition indicated that both estrone sulfate and DHEAS are transported into the cells by the same transport system. In contrast, the uptake of [^{14}C] taurocholate was not inhibited by unlabeled estrone sulfate and DHEAS (Fig. 8*B*), suggesting that taurocholate and estrone sulfate do not share the same transport pathway.

Regulation.

After identification of the existence of a carrier-mediated system for organic anion uptake by HRP-1 cells and the characterization of its nature, we examined the possible regulation of the function of this carrier (Fig. 9). Forskolin activates

adenylyl cyclase, and treatment with this drug has been shown to down-regulate riboflavin uptake in placental BeWo cells (13). 8-Bromo-cAMP (8-Br-cAMP), a membrane-permeable cAMP analog, has been demonstrated to stimulate the expression of the placental facilitative glucose transporter-1 (23). Incubation of HRP-1 cells with 100 μM forskolin or 250 μM 8-Br-cAMP for 3 h resulted in a significant increase in [^{3}H] estrone sulfate uptake. This increased uptake was further enhanced in the presence of 3-isobutyl-1-methylxanthine, which prevents degradation of cAMP by inhibiting cyclic nucleotide phosphodiesterase.

Discussion

During pregnancy, the placenta provides a barrier separating the maternal and fetal compartments. Transporters differentially expressed in the maternal-facing apical membrane and fetal-facing basolateral membrane of the placenta perform the critical task of supplying nutrition from the mother to the developing fetus and eliminating wastes from the fetus. However, many xenobiotics, including therapeutic agents, environmental pollutants, and toxins, bear structural similarity to physiological substrates and have the potential to be recognized by the transporters. Therefore a full understanding of the transport process both in the apical (maternal-facing) and basolateral (fetal-facing) membranes of the placenta and the transporters involved is of clinical, pharmacological, and therapeutic importance.

The present study reports the existence of an organic anion transport system with high affinity for estrone sulfate and DHEAS in the apical membrane of HRP-1 cells. Supporting evidence for the presence of such a transport system include *1*) saturable uptake kinetics (Fig. 2), *2*) significant temperature dependence for the uptake of estrone sulfate (Fig. 3), and *3*) inhibition of uptake in the presence of structural analogs (Fig. 8).

The transport function is Na^+ dependent and Li^+ tolerant (Fig. 4). Replacement of Na^+ by choline led to a significant decrease in estrone sulfate uptake. In contrast, replacement of Na^+ by Li^+ completely restored the stimulatory effect by Na^+. Na^+-dependent and Li^+-tolerant transport has been reported for transporters such as N-system amino acid transporters (5) and phosphate transporters (29).

However, to our knowledge, such a transport system has not been described for organic anion transporters.

Na^+-K^+-ATPase was previously reported to be present in human placenta (28). Our result (Fig. 5) showed that treatment of cells with ouabain, a specific inhibitor of Na^+-K^+-ATPase, inhibited estrone sulfate uptake, suggesting that a Na^+ gradient, established by Na^+-K^+-ATPase, is required for the transport function.

We showed that increasing the H^+ concentration in the incubation medium by lowering the incubation buffer pH led to a marked increase in estrone sulfate uptake (Fig. 6). Several possible mechanisms could explain the observed phenomenon. For example, the effect of pH on estrone sulfate uptake may represent the existence of an estrone sulfate/H^+ cotransport mechanism. A Na^+/H^+ exchanger is known to be present in the placenta membrane (7), which maintains an inwardly directed H^+ gradient. To clarify this assumption, we examined the effect of amiloride, a specific inhibitor of Na^+/H^+ exchanger, on estrone sulfate accumulation. As clearly shown in Fig. 7*A*, pretreatment of cells with amiloride failed to block the stimulation of estrone sulfate uptake caused by lowering the medium pH. Furthermore, pretreatment of cells with FCCP (Fig. 7*B*) and nigericin (Fig. 7*C*), two reagents that disrupt the H^+ gradient across the membrane, was unable to block the increase of the estrone sulfate uptake in the presence of an H^+ gradient. These results suggest that proton transport is not directly involved in estrone sulfate uptake. It is then possible that the stimulation of estrone sulfate uptake at reduced pH (pH 5.0) may arise from a higher rate of nonionic diffusion due to a lower degree of dissociation of estrone sulfate. However, estrone sulfate

is a sulfuric acid monoester. The acidic dissociation constant for such a compound is <0. It can be calculated that, at pH 5.0, almost the entire estrone sulfate is in its dissociated form. Hence, it seems unlikely that the stimulation of estrone sulfate uptake can be attributed to an increased diffusion of an undissociated/un-ionized form. Therefore, the increase in estrone sulfate uptake at a reduced pH might be explained by a direct effect of pH on the estrone sulfate uptake carrier.

Our studies on substrate specificity using radiolabeled compounds (Fig. 8, *A* and *B*) demonstrated that the estrone sulfate and DHEAS are transported into the cells by this system. In humans, DHEAS represents the major circulating steroids secreted by the adrenal cortex. DHEAS not only serves as a precursor for endogenous estrogen synthesis, which is important for continuation of a pregnancy, but also exerts significant neuropsychiatric effects (21, 33). DHEAS induces an expressed feeling of remarkable psychological and physical well-being, notably with improved quality of sleep, greater energy, and an increased ability to handle stress. DHEAS at high blood concentrations, however, shows undesirable effects on the fetus (e.g., intrauterine growth retardation) (27). Estrone sulfate and DHEAS are common substrates for a group of OATPs. They are also the substrates for OAT3 and OAT4, two members of the OAT family. Leazer and Klaassen (19) recently showed with an RT-PCR approach that mRNAs for these multispecific OATs are expressed at various abundances in rat placenta, although whether or not any of these genes produces a functional transport protein in this organ is not known. However, the functional characteristics of the transport system described in the present studies seem to rule out the possibility of the involvement

of these transporters for the following reasons. First, these OATs mediate Na^+-independent transport of their substrates. We showed that the transport of estrone sulfate was Na^+ dependent. Second, taurocholate, a common substrate for members of the OATP family (11), and PAH, a substrate for OAT3 (34), failed to inhibit the estrone transport (Fig. 8*A*). Furthermore, the uptake of radiolabeled taurocholate was not inhibited by unlabeled estrone sulfate and DHEA (Fig. 8*B*), suggesting that taurocholate and estrone sulfate do not share the same transport system described in the present study. OATP-B and OAT4 have recently been localized to the basolateral membrane of human placenta (31), which is different from the apical transport activity observed in our study. The rat isoform of OATP-B is Oatp9. It would be interesting to explore whether Oatp9 or rat OAT4 (not yet cloned) is involved in the basolateral uptake/exit of organic anions in HRP-1 cells. Therefore, the transport system characterized in the present study appears to be different from the known OATP and OAT transporter families described above. The identity of the rat placental cell OAT(s) needs to be investigated further.

In this study, we also provided evidence that intracellular cAMP stimulated transport activity. There are several possible mechanisms that could contribute to the stimulation of the transport activity. For example, it could result from an insertion of transporter proteins (preformed in the intracellular vesicles) into the plasma membranes. Many receptors, transporters, and channels are regulated by this mechanism in response to stimuli (4, 8, 15, 18, 25). The stimulation of transport activity could also result from an increased affinity of the transporter for its substrates due to the conformation change of the transporter, which could be

brought about through its phosphorylation by cAMP-dependent protein kinase pathways. Further studies are needed to differentiate between these possibilities.

In summary, we identified an organic anion transport system on the apical membrane of HRP-1 placental cells, which seems to be different from the previously described organic anion transporters from OATP and OAT families. This transport system appears to be regulated by cellular cAMP levels.

Acknowledgments

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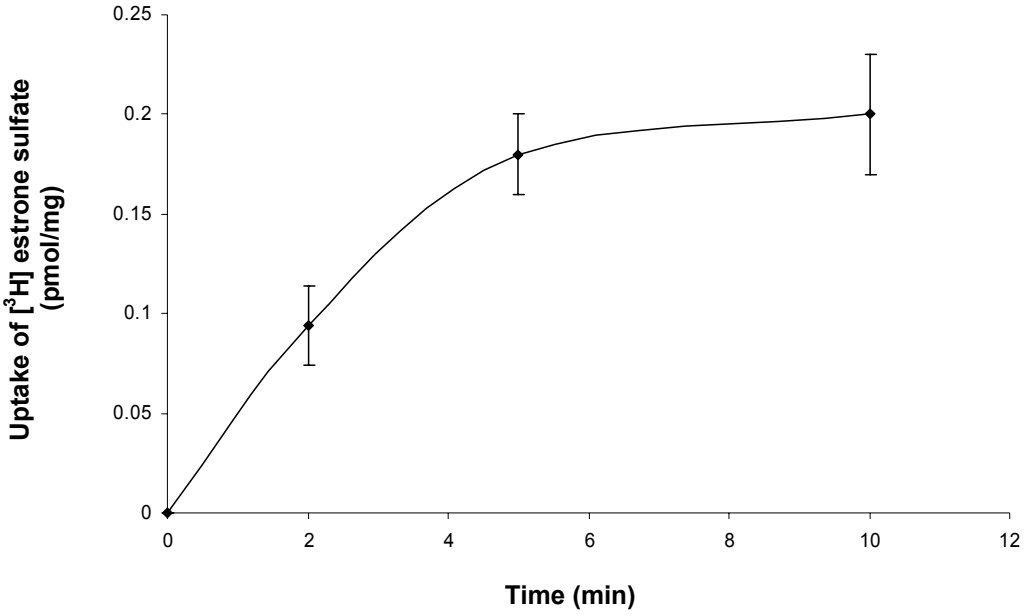


Fig 1. Time course of [^{3}H] estrone sulfate uptake in HRP-1 cells.

Cell monolayers were grown on collagen-coated transwells. [^{3}H] estrone sulfate (50 nM) was added to the apical chambers of the transwells. At specified time points, cells were washed twice with ice-cold PBS, lysed with 0.2 N NaOH, neutralized with 0.2 N HCl, and measured for radioactivity. Each value represents the mean ± SD of 3 experiments.

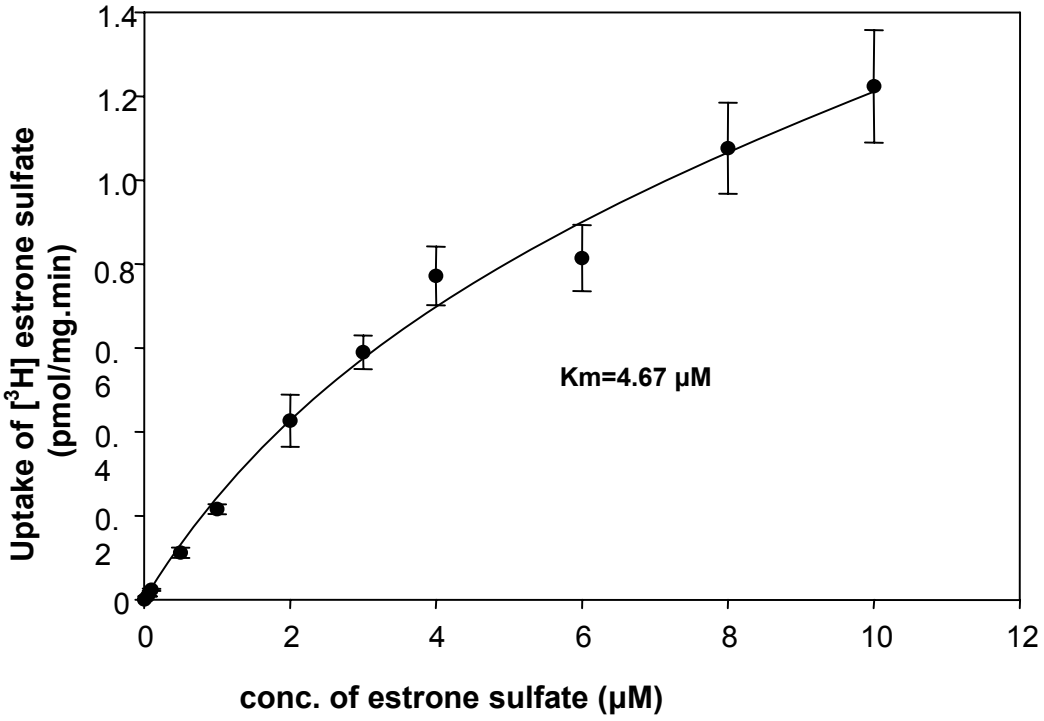


Fig 2. Concentration dependence of estrone sulfate uptake in HRP-1 cells. Cell monolayers were grown on collagen-coated transwells. [^{3}H]estrone sulfate (0.05–10 µM) was added to the apical chambers of the transwells and incubated for 3 min. Cells were then washed twice with ice-cold PBS, lysed with 0.2 N NaOH, neutralized with 0.2 N HCl, and measured for radioactivity. Each value represents the mean ± SD of 3 experiments. Curve represents the calculated fit of the data to *Eq. 1* as described in MATERIALS AND METHODS.

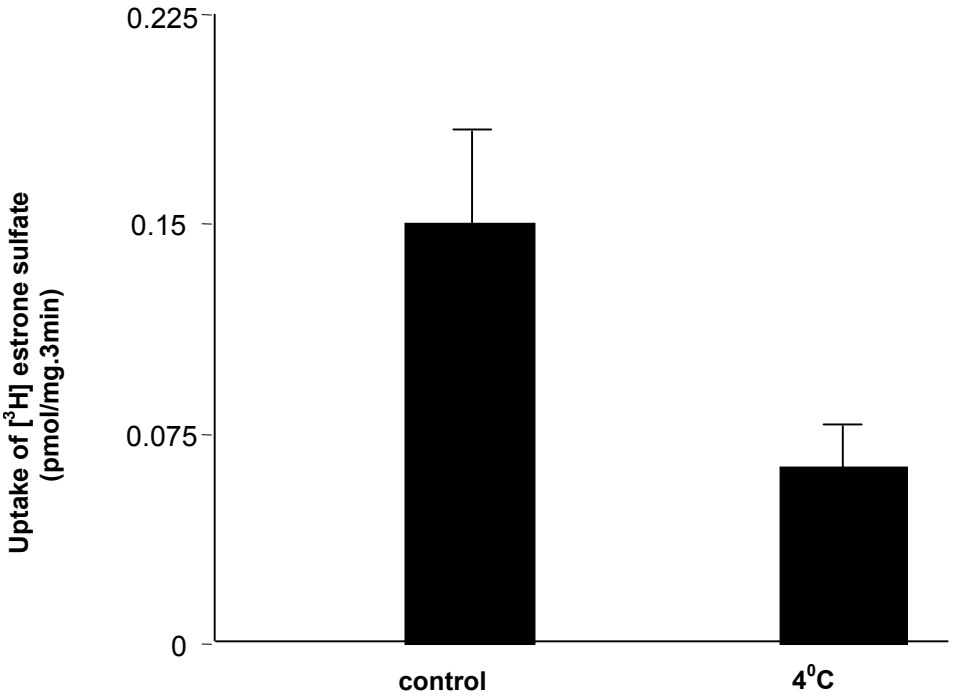


Fig 3. Effect of decreased temperature on apical uptake of estrone sulfate. Apical uptake of [^{3}H]estrone sulfate (50 nM) after a 3-min incubation was measured at both room temperature and 4°C. Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

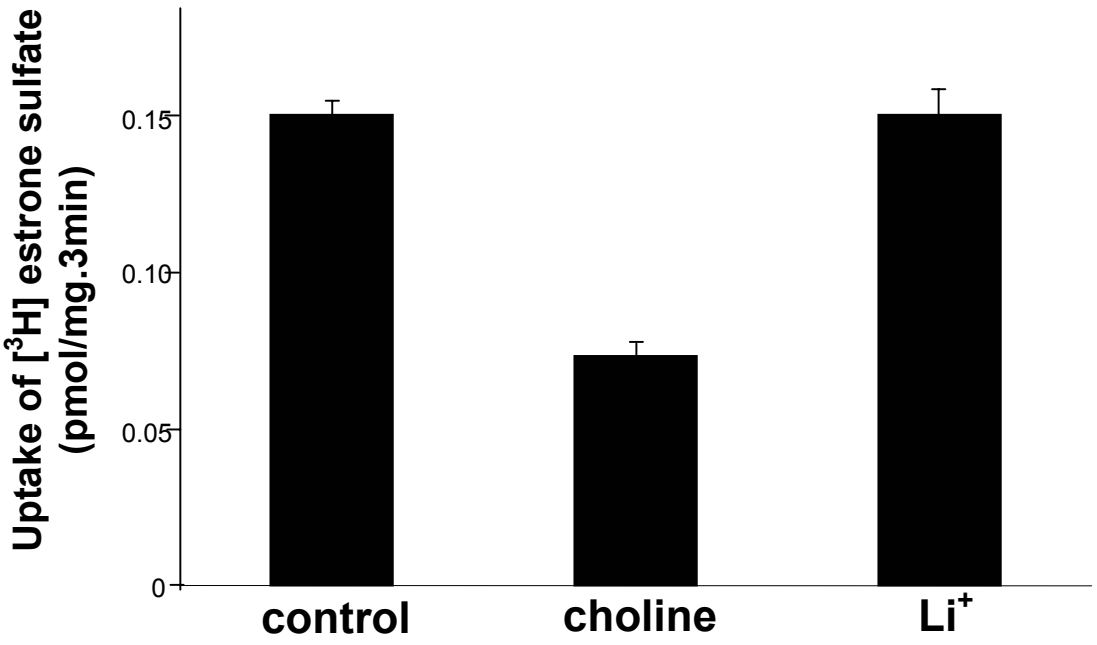


Fig 4. Effect of extracellular Na^+ on uptake of estrone sulfate.

Apical uptake of [^{3}H]estrone sulfate (50 nM) after 3-min incubation was measured in the presence or absence of Na^+ (Na^+ was replaced by choline or Li^+). Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

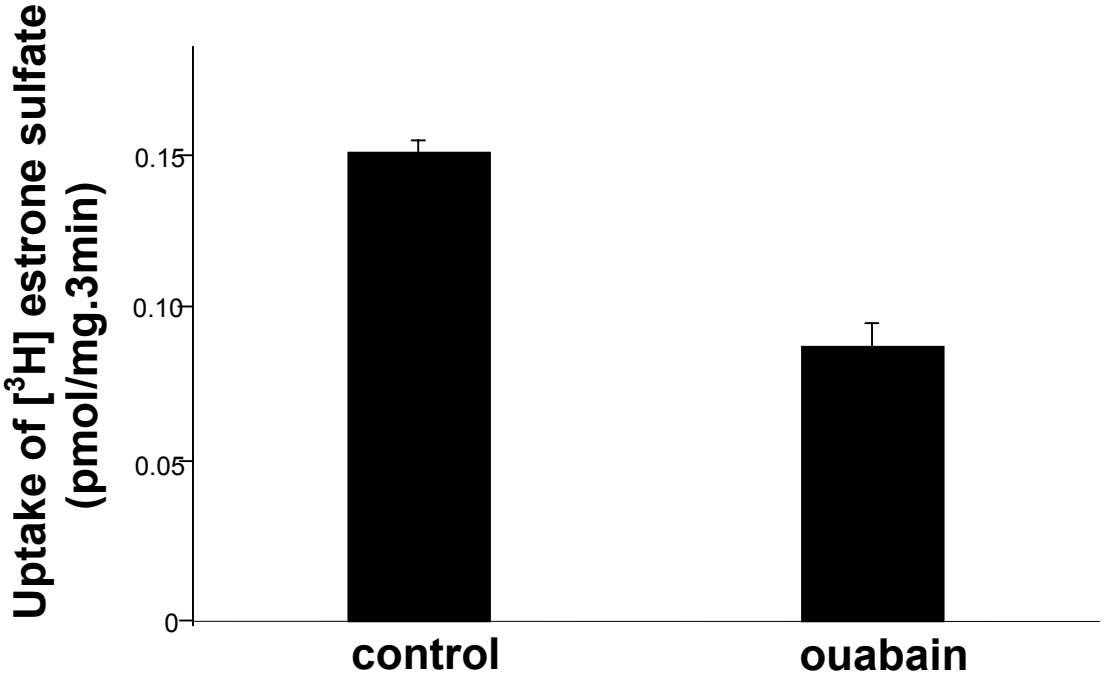


Fig 5. Effect of ouabain on uptake of estrone sulfate.

Cells were incubated with 1 mM ouabain for 1.5 h, and then uptake of [^{3}H]estrone sulfate (50 nM) was measured. Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

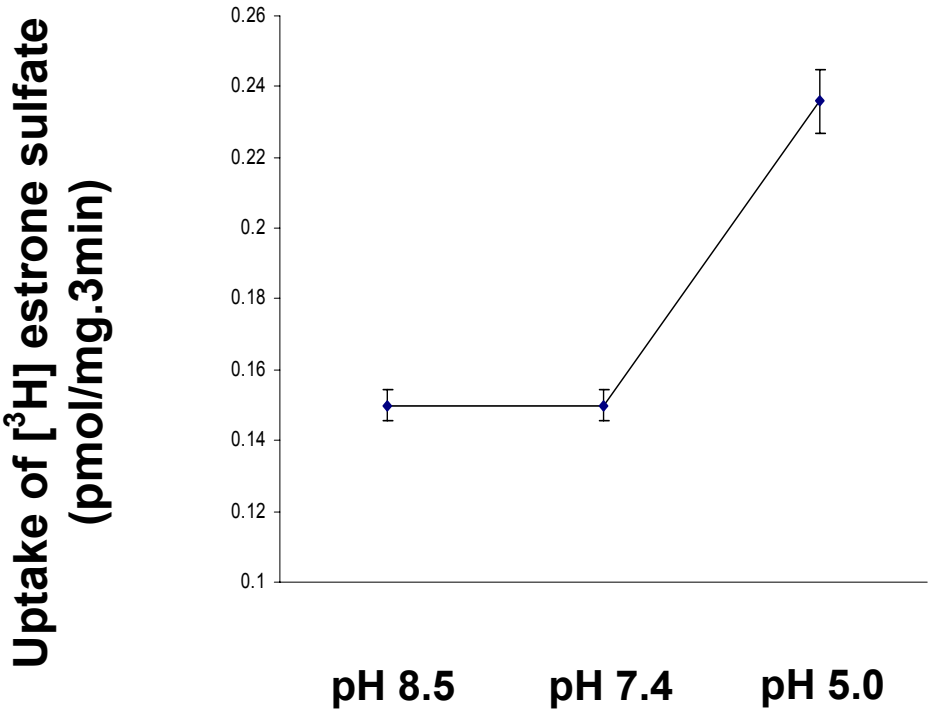


Fig 6. Effect of incubation buffer pH on uptake of estrone sulfate.

Uptake solutions were adjusted to pH 5.0, 7.4, and 8.5, and 3-min apical uptake of [^{3}H]estrone sulfate (50 nM) was then measured. Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

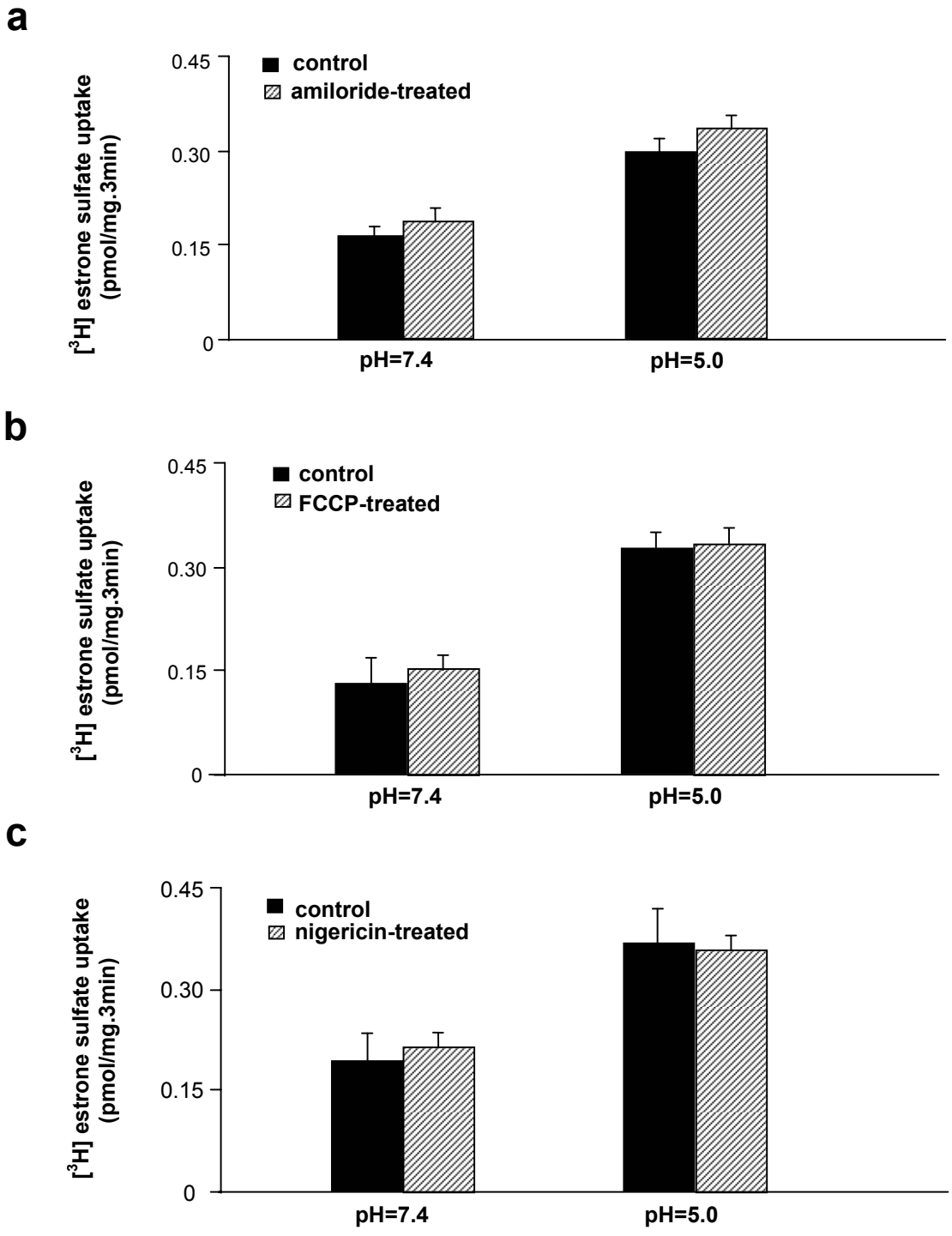
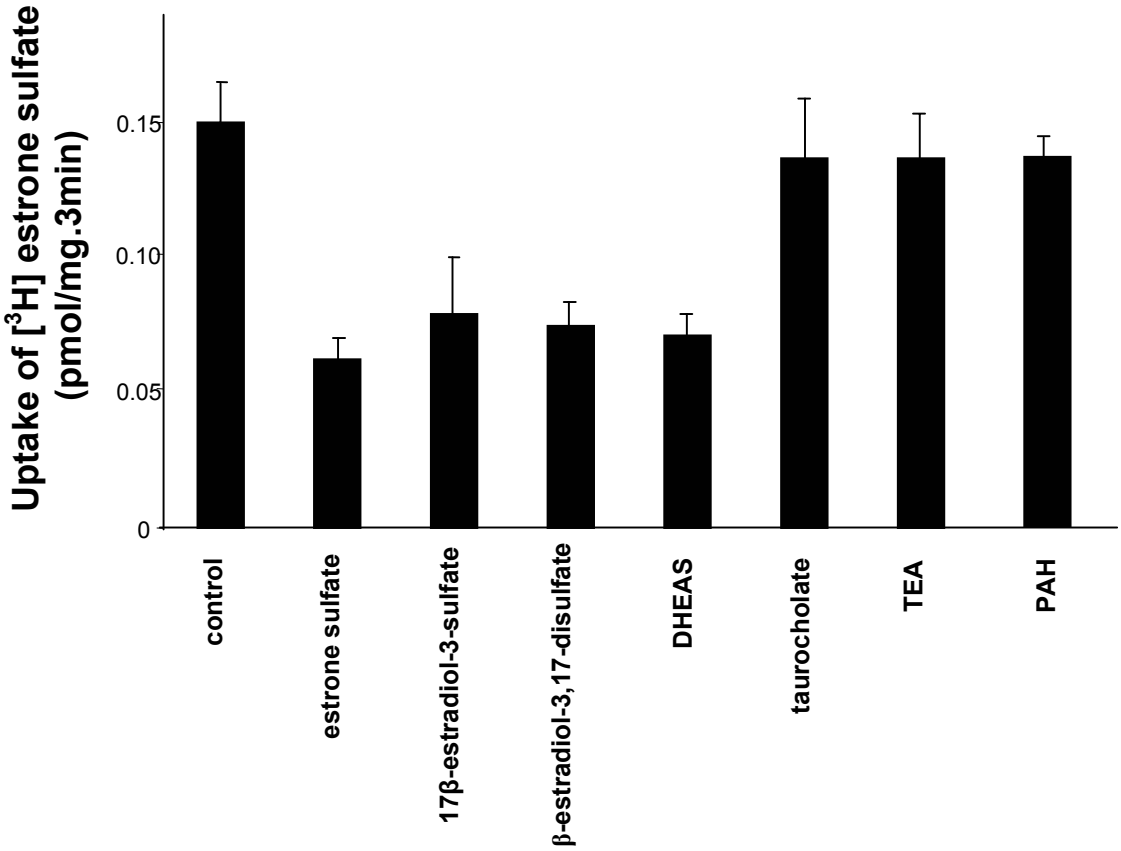


Fig 7. Effects of proton gradient on uptake of estrone sulfate.

Cells were preincubated for 1 h with 1 mM amiloride (*A*), 50 μM carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP; *B*), and 2.5 μM nigericin (*C*), and

uptake of [^{3}H]estrone sulfate (50 nM) was measured at pH 7.4 and 5.0. Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

a


Uptake of [3H] estrone sulfate (pmol/mg.3min)
0.15
0.10
0.05
0
control
estrone sulfate
17β-estradiol-3-sulfate
β-estradiol-3,17-disulfate
DHEAS
taurocholate
TEA
PAH

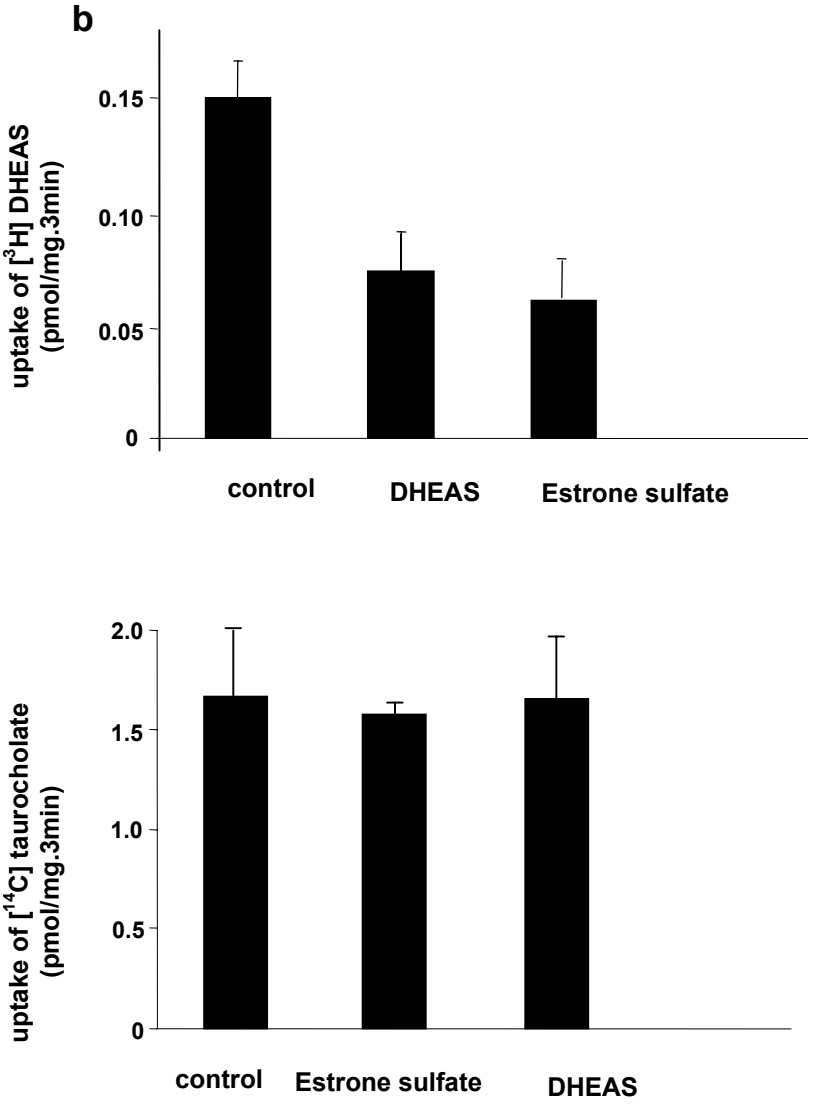


Fig 8. Substrate specificity.

a: inhibition of estrone sulfate uptake by unlabeled organic anions: DHEAS, dehydroepiandrosterone 3-sulfate; TEA, tetraethylammonium; PAH, *p*-aminohippurate. Uptake of 50 nM [^{3}H]estrone sulfate was measured in the presence of 50 μM unlabeled organic anions.

b, top: uptake of [^{3}H] DHEAS (50 nM) in the presence of 50 μM unlabeled estrone sulfate or DHEAS; *bottom*: uptake of [^{14}C] taurocholate (1 μM) in the presence of 1

mM unlabeled estrone sulfate or DHEAS. Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

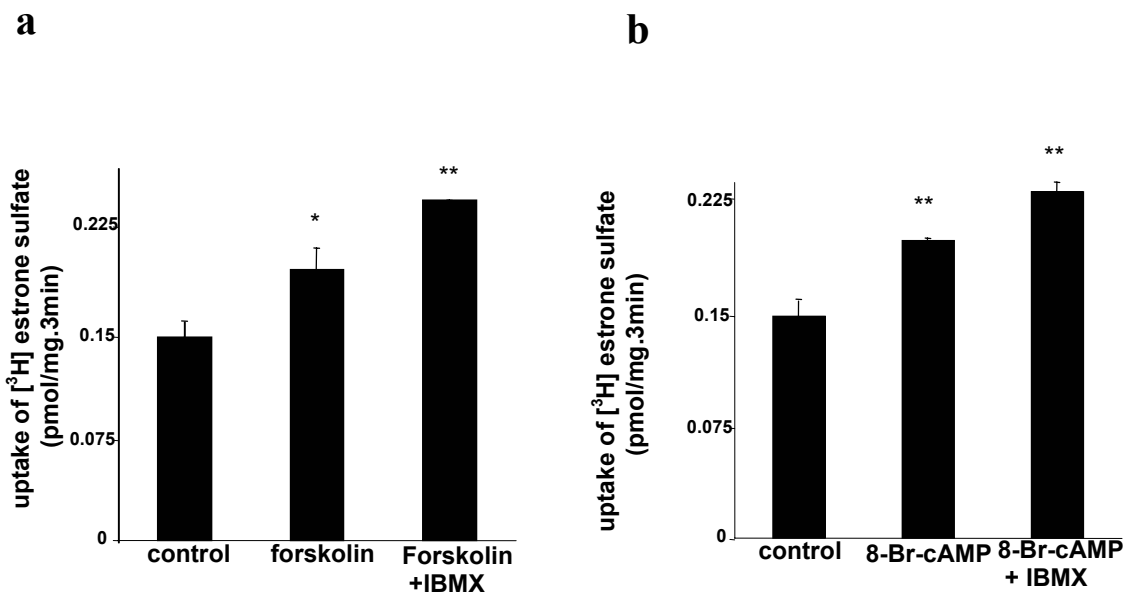


Fig 9. Effect of forskolin and 8-bromo-cAMP (8-Br-cAMP) on estrone sulfate uptake.

Cell monolayers were preincubated for 3 h with 100 μM forskolin (*A*) or 250 μM 8-Br-cAMP (*B*) with or without 1 mM IBMX, and then apical uptake of [^{3}H]estrone sulfate (50 nM) was determined. Each value represents the mean ± SD of 3 experiments (*P < 0.05 and **P < 0.01 vs. control).

Summary

Organic anion transporter family (OAT) is a family of cell membrane transporter proteins that responsible for the absorption, distribution, and excretion of a diverse array of environmental toxins, and clinically important drugs including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories. Therefore they are critical for the survival of mammalian species.

My research work has explored the important roles of protein glycosylation, critical residues, protein kinases and hormone as well as protein-protein interaction on maintaining OAT transport functions. Furthermore, my studies have characterized two useful *in vitro* placental cell models, which can be used in the future studies of other transporter proteins expressing in human placenta. My studies on OAT structure-function relationship and regulation are helpful to us in better understanding certain human diseases and developing novel clinical drugs.

Since the *in vivo* situation will be more complicated and we still don't know whether it will give us the same results when conducting the same studies under *in vivo* conditions, it will be great interests for us to precede the studies using *in vivo* model and further explore the structure-function relationship and regulation of OATs in the future.

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Research Publications

1. ***Zhou F***, Xu W, You G., Comparison of the interaction of human organic anion transporter hOAT4 with PDZ proteins between kidney cells and placenta cells. Pharm. Res. 2007 Jun 30; [Epub ahead of print]

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Book Chapter

Zhou F, and You G., The role of membrane transporters in drug disposition (Drug Transporters (tentative), ed. Han C. and Wang B.) to be published, 2007, Wiley & Son, New York, NY